TRANSCRIPTIONAL ANALYSIS OF CHICKEN IMMUNE CELLS FOLLOWING EXPOSURE TO 2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN (TCDD)

A Dissertation

by

NAHUM PUEBLA-OSORIO

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2004

Major Subject: Poultry Science

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ABSTRACT

Transcriptional Analysis of Chicken Immune Cells Following Exposure to 2,3,7,8-Tetrachlorodibenzo-p-Dioxin (TCDD). (December 2004)

Nahum Puebla-Osorio, D.V.M., National Autonomous University of Mexico (UNAM); M.S., Texas A&M University

Chair of Advisory Committee: Dr. Luc R. Berghman

In the present investigation, microarray analysis was used to identify potential TCDD gene targets. Three microarray experiments were performed to study the effect of TCDD in an established chicken B-cell line (DT40), in a chicken macrophage cell line (HD11), and in the bursa of Fabricius from embryos exposed in ovo at 6 days of incubation. From the DT40 microarray analyses, clones with sequence similarity to the apoptotic genes caspase 8 and caspase 9, and the transcription factor NFκB, among others, were identified. Real-time quantitative polymerase chain reaction (RT-PCR) revealed that TCDD elicits aryl hydrocarbon receptor (AhR)-mediated apoptosis in the avian DT40 pre-B-cell line through activation of caspases 9 and 3 (see chapter III). During the course of the HD11 microarray analyses, a consistent down-regulation of the matrix metalloprotease MMP-2 was observed. This finding was the basis for the hypothesis that TCDD has an effect on the gene expression of the MMP-2 and MMP-9 in macrophages. Then, gene expression analysis and functional zymography showed that TCDD impairs the MMP-2 and MMP-9 response to LPS stimulation in HD11 chicken macrophages (see chapter V). The microarray analyses of the embryonic bursa

of Fabricius provided the basis to further study of the effect of TCDD in the chicken embryo. The shifted genes were classified according to their function. The down-regulated genes included: precursor of matrix metalloprotease-inhibitor, histone acyl-transferase 1, homeobox protein CUX-2, Death Associated Protein Kinase, and UDP-glucosyl transferase, among others. The up-regulated genes included: phosphoinositide-specific phospholipase, acyl Co-A oxidase, and protein effector of Cdc42, among others.

Together, these microarray analyses produced a database of genes of interest that will provide sufficient hypotheses to inspire multiple investigations aimed at confirming and refining the gene expression alterations as a consequence of TCDD exposure.

DEDICATION

To my parents

Mr. German Puebla and Mrs. Soledad Osorio

and my brothers and sisters.

To the memory of my brothers German and Leonel.

ACKNOWLEDGEMENTS

I am deeply grateful to Dr. Luc R. Berghman, my advisor, for allowing me to work with him during both my M.S. and Ph.D. degrees. His patience, wisdom, and broad academic knowledge combined with a great sense of humor makes him an unparalleled human being. Dr. Berghman has been the cornerstone of my success during my graduate studies. The knowledge and experience that I have gained from him will be cherished forever.

I am grateful to Dr. Ken Ramos for his support throughout my research; it would not have been possible to accomplish it without his help. His advice was key in the development of this project. I also appreciate having him as committee member and dissertation advisor; his support and wise advice will always be remembered.

I thank Dr. Ian Tizard and Dr. James Zhu for serving as my committee members and dissertation advisors.

My appreciation goes to Dr. Mary Walker and Irina Ivnitski-Steele, from the Department of Pharmacy of the University of New Mexico for all their help on the *in vivo* assay. Dr. Walker provided resources to accomplish part of this project.

I thank Ms. Daad Abi-Ghanem, my lab mate and good friend, for helping me run several real-time assays. Thanks to Dr. Nancy Ing and Cindy Balog for their friendship and all the help that I received when doing lab work. Thanks to Dr. Laurie Davidson for letting me "break into" her lab during weekends to use their equipment.

My appreciation goes to Dr. Luis Martinez-Lemus, his friendship and support are highly regarded.

I thank Dr. Roger Smith for his help on flow cytometry assays. Thanks to Dr. Dick Creger and Dr. Alan Sams for all their support and care during my studies.

I thank Dr. Joan Fleming for helping me every time I looked for help from her.

My great appreciation goes to Dr. Michela Gentile, I will never forget her friendship and continuous support during my studies at Texas A&M University; without her help it would have been very difficult to cope with the adversities I had to face during my Ph.D. program. I feel fortunate to have her as my best friend.

My appreciation also goes to my best friend, Ricardo Jimenez Perez, for his support and long lasting friendship.

My appreciation is extended to my friends at Texas A&M University, and some now away in different endeavors in different parts of the world: Uwe Schneider, Martha Leon, Jeff Bryan, Ken Widmer, Saravjit Rihal, Marcelo Aguiar, Veronica Manrique, Francisco Abello, Kelly Higgins, Azza Al-Zein, Luis Francisco-Revilla, Erwin Hesse, and Mario Villarino. Thanks to Jennifer Farmer for her help and support in this last year. It has been an unforgettable time at Texas A&M University in College Station.

I would like to acknowledge my parents, German Puebla and Soledad Osorio. My mother has been the backbone of my success. I am infinitely grateful to her for the years of hard work. I am also grateful to my sisters, Elvia, Olga and Martha; I will always remember their loving support.

I thank God for giving me the strength and wisdom to explore the world of science, and for giving me the opportunity to enjoy and understand life.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

TCDD in Mammalian Species

The polycyclic hydrocarbons such as the halogenated hydrocarbons (TCDD, PCBs and PBBs) and aromatic hydrocarbons (benzopyrene, DMBA), are ubiquitous environmental chemicals that have been characterized as both potent immune-toxicants and carcinogens (Abbott et al., 1995). 2,3,7,8-tetra-chlorodibenzo-p-dioxin (TCDD) (Fig. 1.1), the classic dioxin, causes a myriad of biological effects that are mediated almost entirely through the activation of the aryl hydrocarbon receptor (AhR) (Schmidt and Bradfield, 1996). The AhR contains the basic helix-loop-helix (bHLH) and PAS (Per/ARNT/Sim) domain (Whitlock, 1999). Its inactive form is found in the cytoplasm as a tetrameric complex composed of the ligand binding subunit, a dimer of the 90-kDa heat shock protein, and X-associated protein 2 (Lees et al., 2003). Upon binding to an agonist such as TCDD, the AhR translocates to the nucleus and heterodimerizes with the aryl hydrocarbon receptor nuclear translocator (Arnt) (Carver and Bradfield, 1997; Hoffman et al., 1991).

This dissertation follows the style of *Immunity*.

The protein heterodimer complex binds to dioxin-responsive elements (DRE) located upstream of the regulatory regions of target genes that include cytochrome P450 isoform 1A1 (CYP1A1), CYP1A2, CYP1B1, and NAD(P)H:quinone oxidoreductase (Hankinson et al., 1991; Whitlock, 1999) (Figure 1.2).

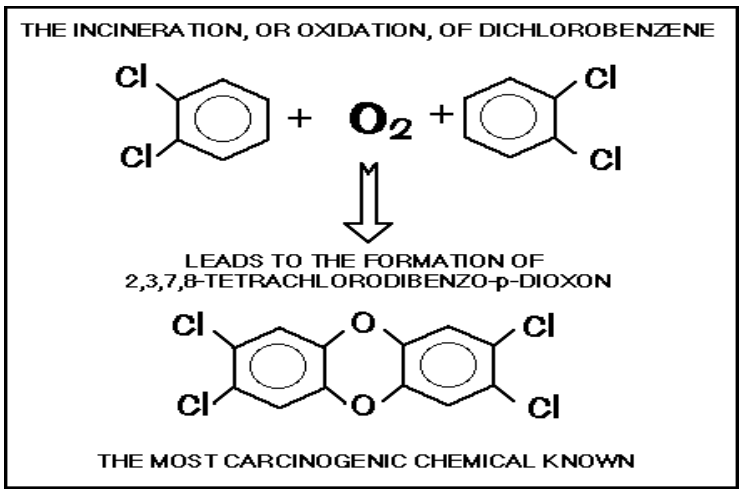

Figure 1.1. 2,3,7,8-tetrachlorodibenzo-p-dioxin results from the incineration of Dichlorobenzene and other compounds (www.toxicspot.com/ ejpp/ejppv36.html).

The AhR repressor (AhRR), a PAS protein, inhibits AHR signal transduction by competing with AhR for Arnt and also by binding to DRE; the repressor is induced by the AhR, leading to a negative feedback loop for its regulation (Mimura et al., 1999); (Mimura and Fujii-Kuriyama, 2003).

The AhR signal transduction pathway involves protein kinase C and tyrosine kinase, since inhibitors of these kinases block the induction of target genes (Berghard et al., 1993; Carrier et al., 1992; Gradin et al., 1994; Kikuchi et al., 1998). Several

investigators have studied the transcriptional profile of a wide variety of cell types, showing alterations in the expression of a number of known genes as a consequence of exposure to TCDD. Cell lines that have been studied include human HepG2 cells (Puga et al., 2000), Hepa1c1c7 cell line (Jin et al., 2004), non-tumorigenic (HPL1A) and tumorigenic lung cell lines (A549) (Martinez et al., 2002), thymocytes (Fisher et al., 2004) and hepatocytes from CA-AhR-transgenic mice (Moennikes et al., 2004).

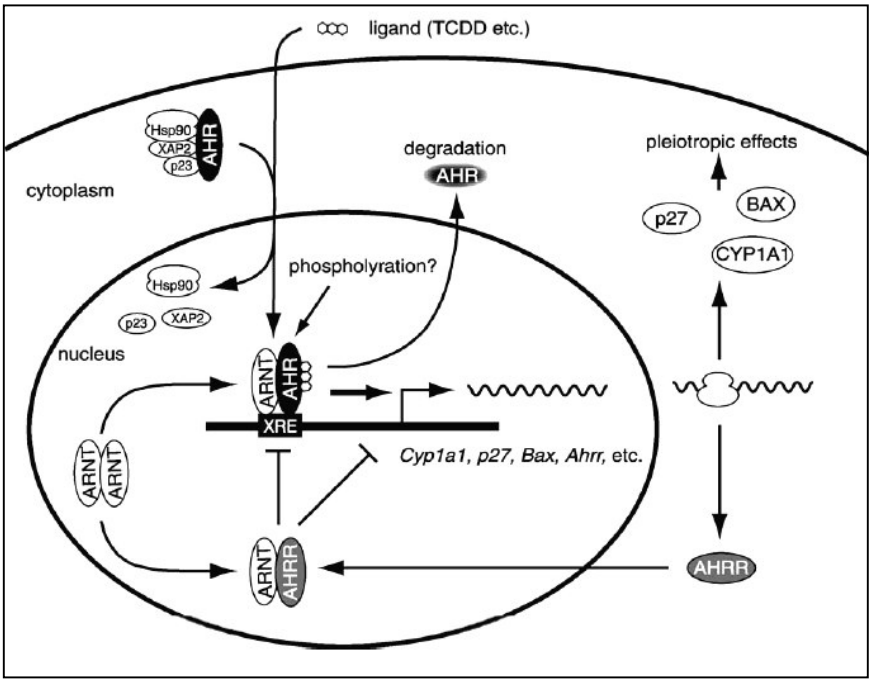

Figure 1.2. Mechanisms of transcriptional activation by AhR and negative feedback regulation of AhR by AhRR (Mimura and Fujii-Kuriyama, 2003).

The genes that were influenced by exposure to TCDD were classified according to their functions, which included genes involved in regulation of the cell cycle,

differentiation, apoptosis, inflammation, development, cell adhesion, tumorigenesis and genes coding for transcription factors. In addition, genes that have been reported to contain dioxin-responsive elements (DRE) in their promoter region include metabolic enzymes (Nebert, 1994), growth factors (Kim et al., 2000; Zaher et al., 1998) and cytokines (Lai et al., 1996). A strong correlation has been established between exposure to high doses of TCDD and certain types of cancer and cardiovascular diseases in accidentally exposed populations (Bertazzi, 1991; Fingerhut et al., 1991). In mice, a number of reports have shown that exposure to TCDD during development leads to hydronephrosis (Birnbaum, 1995; Couture et al., 1990b) and cleft palate (Abbott et al., 1995; Couture et al., 1990a). TCDD also causes disorders of lipid metabolism as well as cardiovascular and craniofacial teratogenesis (Guiney et al., 1997; Hornung et al., 1999), immunotoxicity (Kerkvliet, 1995; Oughton et al., 1995), disruption of the endocrine system, chloracne (non-inflammatory keratinization of pilosebaceous units with comedones and dilated infundibulum after exposure to aromatic chlorinated hydrocarbons of various structures), and reproductive dysfunction (Bjerke and Peterson, 1994; Kim et al., 2000; Mably et al., 1992; Zaher et al., 1998).

The disruptive effects of TCDD on the immune system (reviewed by (Holsapple et al., 1991), show increased AhR expression upon immune activation; this increase of the AhR has been documented in macrophages (Hayashi et al., 1995), B-cells (Marcus et al., 1998) and T-cells (Lawrence et al., 1996). Thus, immune activation of leukocytes (Kerkvliet, 2002) may confer an enhanced sensitivity to TCDD toxicity. In addition, TCDD exerts direct suppression of the humoral immunity by inhibiting IgM secretion by

B-lymphocytes during differentiation, with only modest effects on B-cell proliferation (Dooley and Holsapple, 1988; Luster et al., 1988; Wood and Holsapple, 1993). The ability of TCDD to suppress the IgM secretion is associated with the presence of a DRE within the Ig heavy chain 3'alpha-enhancer, as reported in the CH12.LX B cell line (Sulentic et al., 1998; Sulentic et al., 2000). While immunosuppression by TCDD is believed to be primarily mediated through the AhR, other studies have suggested that TCDD is capable of inducing apoptosis in T-cell lines in an AhR-independent process following upregulation of pro-apoptotic genes (Hossain et al., 1998). TCDD has also been shown to induce apoptosis in AhR-deficient El-4 mouse thymoma cells (Park et al., 2003).

Embryonic Development of the Avian Immune System

The bursa of Fabricius is the primary lymphoid organ of the humoral immune system and is considered as Gut-Associated Lymphoid Tissue (GALT) (Glick, 1995). It arises as a dorsal diverticulum of the proctodeal region of the cloaca between days 3 and 5 of embryonic life (Olah et al., 1986); (Romanoff, 1960). About 11 to 14 primary folds (plicae) and 6 to 7 secondary plicae develop from embryonic day 9 through the first week post-hatch. The prebursal lymphoid stem cells that will later develop into mature B-lymphocytes are believed to colonize the bursa between day 8 and 14 of embryogenesis (Ratcliffe, 1989). Each follicle is initially populated by two to five rapidly dividing B-cell precursors containing productive Ig gene rearrangements. During the embryonic phase of bursal development, B-cell numbers nearly double every

24 hours. Just before hatch, B-cells expressing surface immunoglobulin (sIg) form a cortex containing small, densely packed lymphoid cells around the medulla of each follicle. The embryonic thymus is populated with three separate waves of stem cells: days 6-8, days 12-14, and days 18-21 of embryonic development (Dunon and Imhof, 1996). The cell phenotypes of the thymus and their ontogeny are very similar in chickens and in mammals. T-cells mature within the thymus while undergoing a series of changes of their localization and their phenotype (Chen et al., 1996).

TCDD and the Immune System in Avian Species

During the prenatal period, the developing immune system is extremely vulnerable to immune-toxicants in mammals, as well as in birds, because of the large number of dynamic changes that then takes place. For instance, toxicant interference with a dynamic process such as B lymphocyte selection in the avian bursa of Fabricius (mammalian bone marrow) is likely to be much more devastating than the same toxicant exposure of a fully matured B-lymphocyte population.

The gross effects of TCDD on the avian immune system have been described extensively; the effects in the bursa of Fabricius have been related to inhibition of lymphoid development, atrophy (Andersson et al., 1991), and decrease of bursal weight (Powell et al., 1996) in the developing chicken bursa of Fabricius. Other reports have described the reduction of both bursa of Fabricius and thymus weights after in ovo administration of TCDD and 3,3',4,4',5-pentachlorobiphenyl (PCB126) (Fox and Grasman, 1999). It is suggested that the inhibition of lymphoid development in the

bursa is caused by the direct effect of TCDD in the bursal microenvironment, resulting in a decrease in the attraction of stem cells and/or in the capacity to induce proliferation of the colonizing cells (Nikolaidis et al., 1990); and a suppression of B-cell proliferation in hatchlings from TCDD-injected eggs has been observed in a dose-dependent manner (Peden-Adams et al., 1998).

The underlying gene interactions that mediate the observed phenomena have not been clarified to date. The chicken AhR basic helix-loop-helix and the PAS domains exhibit more than 90 % and 85 % sequence similarity respectively, with that of the mammalian Ah receptor (Walker et al., 2000). In addition, these authors showed that the chicken AhR dimerizes with human AhR nuclear translocator and binds to the mammalian dioxin-response element (DRE) in a ligand-dependent manner. The cytochrome P450 isoform 1A4 (CYP1A4), the avian homologue of CYP1A1 (Gilday et al., 1998) was highly induced by TCDD in a subset of tissues expressing AhR. The highly conserved nature of DREs and AhRs at the level of DNA binding was also reported by earlier studies on the species-specific binding of the transformed AhR to the DRE (Bank et al., 1992). These DNA-binding analyses suggested that nucleotide sequence closely related to the murine consensus DRE sequence is responsible for conferring AhR-dependent TCDD responsiveness in species ranging from rats, rabbits and mice to fish and chicken. Given the availability of the chicken genome sequence, it should thus be possible to screen this sequence database for genes containing a consensus DRE sequence in their promoter regions. In theory, each of those genes should be TCDD-responsive in an AhR-dependent manner.

The objective of our study was to provide a comprehensive investigation of the transcriptional profile of the chicken immune cells, including embryonic bursa of Fabricius, when exposed to TCDD. This type of study has never been performed before and may lead to a better understanding of the transcriptional regulatory mechanisms affected by TCDD, and its biochemical and physiological consequences.

Microarray Analysis

Microarray technology has emerged as one of the most important tools to screen and exploit the entire transcriptome of a cell (Lockhart et al., 1996). It is currently one of the most powerful tools for the direct monitoring of large numbers of mRNAs in parallel.

Microarrays have expanded dramatically in genomic research and are applied in diverse fields including immunology (Staudt and Brown, 2000; van der Pouw Kraan et al., 2004), microbiology (Manger and Relman, 2000; Schoolnik, 2002), cancer research (Grant et al., 2004; Li et al., 2002), parasitology (Rathod et al., 2002) and toxicology (Ezendam et al., 2004; Puga et al., 2000), among others.

The spotted cDNA microarray has been the most common method utilized since the mid 90's (Fig. 1.3). This type of microarray consists of numerous probes of PCR-amplified cDNA fragments placed in a known pattern of spots on a treated glass surface, crosslinked and dried. These probes are then targeted with labeled (with either green or red fluorescent dyes) cDNA derived from mRNA (through reverse transcription) extracted from two different cell populations (e.g. treated versus negative control).

The respective labeled samples are co-hybridized on the same glass slide (competitive hybridization). Then the array is scanned at two different wavelengths (635 nm for the red dye and 532 nm for the green dye), producing two 16-bit tiff images, corresponding to the two different conditions, which are then merged for analytical purposes. Some areas of the microarray are left empty for background correction, and the target cDNA should not bind these areas (should not fluoresce).

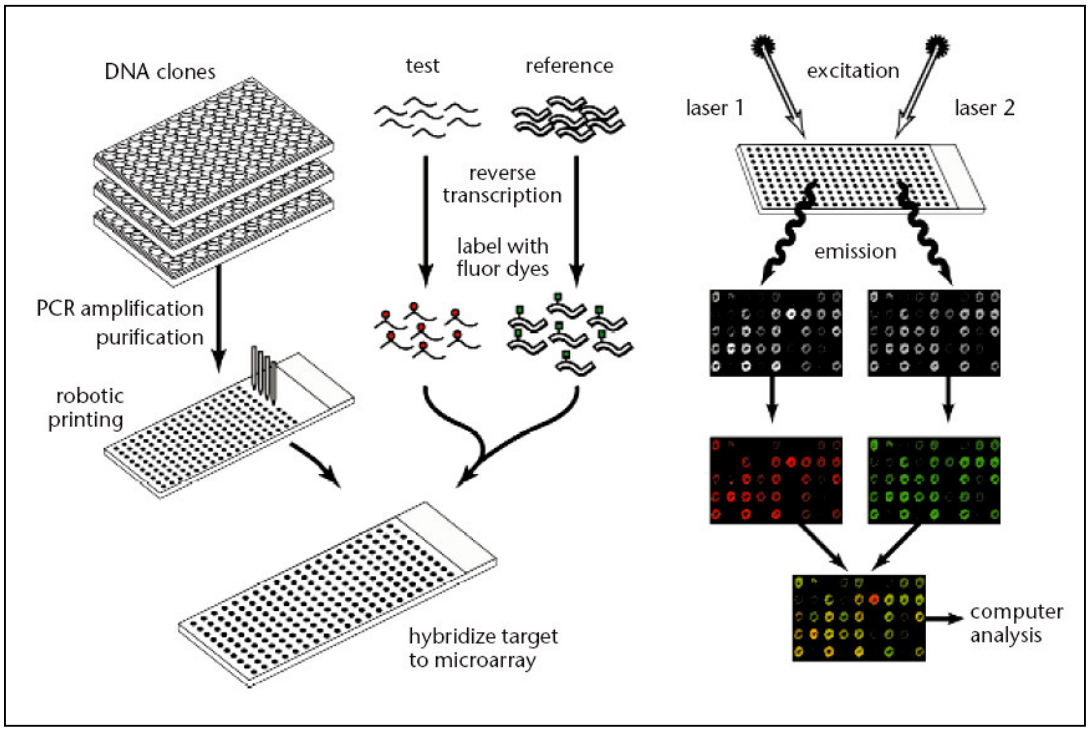

Figure 1.3. The cDNA microarray principle (Duggan et al., 1999).

A more recent, very powerful, microarray technology is the in situ-synthezised oligonucleotide array using photolithographic technology (Affymetrix). This technology

is not based on competitive hybridization. Instead, to compare two samples, two separate microarrays are required (Grant et al., 2004). The background of this technology is that each gene target is probed by a number of distinct probes (11-20).

Theoretically, each probe consists of millions of segments of the exact same length (25 bp) and sequence, arrayed in a small squared area. Thus, biotinylated cRNA derived from a biological sample is hybridized onto the microarray, stained and scanned for fluorescence at a single wavelength. This type of microarrays has become the reference standard, since every advance in the field is compared against this technology.

Agilent's Inkjet technology uses a somewhat different non-contact in situ synthesis process of printing 60-mer length oligonucleotide probes, base-by-base, from digital sequence files. This technology is based on a standard phosphoramidite chemistry used in the reactions that allows high coupling efficiencies to be maintained at each step in the synthesis of the full-length oligonucleotide (Agilent, Palo Alto, CA; http://www.chem.agilent.com).

Finally, Forster et al. (2004) have described a new technology that incorporates alexa 594 as a third dye-label in addition to the commonly used Cy3 and Cy5. These authors argue that the method enables direct comparison between two experimental populations as well as measuring these two populations in relation to a third reference sample, allowing comparisons within the slide and across slides. The authors agreed that the triple-target approach should not be considered as a new standard, but rather as a valuable addition to the existing microarray technology (Forster et al., 2004).

In our own studies, we have used a commercially available high density DNA microarray to analyze the transcriptional profile induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in DT40 B-cells, HD11 chicken macrophages and embryonic chicken bursa of Fabricius. The array was created at the Fred Hutchinson Cancer Research Center (FHCRC). In our in vitro studies of DT40 and HD11 cells, we used the initially produced immune array, which consisted of approx. 4,000 clones. Our more recent in vivo study used an upgraded microarray consisting of 13,007 clones. This array included different sources of chicken cDNA libraries and control features. The annotation file is available at: ftp://milano.fhcrc.org/ArrayLab/chicken13k/annotation/. The microarray was used as a basis for the development of a database of genes whose expression is affected by TCDD. To complement the data analysis, we have used three different clustering methods (GENESIS software; URL: http://genome.tugraz.at) including hierarchical clustering, self-organizing maps and k-means clustering, to facilitate the classification and visualization of differentially expressed genes into sets with similar expression patterns (Dysvik and Jonassen, 2001; Sturn et al., 2002).

In the present investigation, we used microarray analysis to identify potential TCDD gene targets. We performed three microarray experiments to study the effect of TCDD in an established chicken B-cell line (DT40), in a chicken macrophage cell line (HD11), and in the bursa of Fabricius from embryos exposed in ovo at 6 days of incubation. From the DT40 microarray analyses, we identified clones with sequence similarity to the apoptotic genes caspase 8 and caspase 9, and the transcription factor NFκB, among others, as being of particular interest. This led us to pursue further

analyses using real-time quantitative RT-PCR to assess whether the shift observed in the microarray analysis was authentic. From this investigation, we were able to conclude that TCDD elicits aryl hydrocarbon receptor (AhR)-mediated apoptosis in the avian DT40 pre-B-cell line through activation of caspases 9 and 3 (see chapter III). During the course of the HD11 microarray analyses, we observed a consistent down-regulation of the matrix metalloprotease MMP-2. This finding was the basis for the hypothesis that TCDD has an effect on the gene expression of the MMP-2 and MMP-9 in macrophages. We carried out gene expression analyses and functional zymography. From this investigation we concluded that TCDD impairs the MMP-2 and MMP-9 response to LPS stimulation in HD11 chicken macrophages (see chapter V). The microarray analyses of the embryonic bursa of Fabricius provided the basis to further study of the effect of TCDD in the chicken embryo. The shifted genes were classified according to their function. The list of genes of interest included down-regulated genes such as: precursor of matrix metalloprotease-inhibitor, histone acyl-transferase 1, homeobox protein CUX-2, Death Associated Protein Kinase, and UDP-glucosyl transferase, among others. The list of up-regulated genes included: phosphoinositide-specific phospholipase, acyl Co-A oxidase, and protein effector of Cdc42, among others. In a similar manner as demonstrated with DT40 and HD11 cells, this database of genes of interest will inspire future investigations aimed at confirming and refining the gene expression alterations suggested by the microarray analyses. It goes without saying that each single microarray-based study provides enough hypotheses to inspire multiple investigators for years to come.

CHAPTER II

MICROARRAY ANALYSIS OF THE CHICKEN DT40 B-LYMPHOCYTE CELL LINE EXPOSED TO 2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN (TCDD)

Overview

The chicken pre-B-cell line DT40 is widely regarded as a useful genetic model system to study the functions of genes involved in the humoral immune response. In this study, DT40 cells were used in combination with a chicken immune cDNA microarray constructed at the Fred Hutchinson Cancer Research Center (FHCRC) to assess the repercussions of short term exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on the gene expression profiles of avian B-lymphocytes. The complete cDNA chicken immune array contained approximately 4186 transcripts representing about 2,200 different genes.

Cultures of v-rel immortalized chicken B lymphocytes (DT40, ATCC-CRL-2111) were exposed to two different concentrations of TCDD (1 and 10 nM) for 6 and 12 h. Cells challenged with an equivalent volume of dimethyl sulfoxide (DMSO, vehicle), and non-exposed cells were used as controls. In total, twelve 75-cm^2 flasks were used to run each experimental condition in triplicate, including: 1 and 10 nM TCDD for 6 and 12 hours each, vehicle (DMSO) and non-treated cells. The labeling of the cDNAs with Cy-3 and Cy-5, the hybridization of the microarray slides and the scanning of the slides (yielding 16-bit tiff images) were performed by the FHCRC.

Image analysis was performed using GenePix Pro 4.0 (Axon Instruments, Inc.). The log2 of the ratio of median intensities was used for further data analysis. Hierarchical clustering, and tree formation were designed to study and classify the TCDD-responsive genes in the DT40 B cell line (GENESIS, http://genome.tugraz.at/Software/GenesisCenter.html). In the cluster with the most pronounced gene expression profile (cluster C), a trend of up-regulation was observed after 6 h exposure to TCDD, and then a down-regulation was observed after 12 h of exposure. At the 1 nM exposure level, genes that were up-regulated after 6 h and then down-regulated after 12 h of exposure included: TNF-ligand (similar to B-lymphocyte stimulator), cytotoxic granulocyte-associated RNA-binding protein, cytochrome C oxidase (polypeptide I), Cdc25 B-type tyrosine phosphatase, among others. At the 10 nM TCDD exposure level, similar gene expression profiles were observed. Genes that underwent up-regulation after 6 h of exposure, and then showed down-regulation after 12 h of exposure included DNA repair protein RAD 9 and matrix metalloprotease 2 (MMP-2). The multiple leads pointing in the direction of apoptosis related phenomena have led us to pursue the working hypothesis that exposure to TCDD induces a shift in apoptosis-related genes that could ultimately lead to loss of pre-B-cells (see Chapter III).

Introduction

The chicken bursal pre-B-cell line DT40 is a useful genetic model system to study the functions of genes involved in the humoral immune response. DT40 is an avian leukosis virus (ALV) induced bursal lymphoma cell line derived from a Hyline SC chicken. The original lymphoma was induced by viral infection of a 1-day old chicken with Rous associated virus 1 (RAV-1). DT40 cells retain the ability to rearrange the immunoglobulin light chain gene (IgL) (Buerstedde et al., 1990), and they exhibit a lymphoblastoid phenotype (Baba and Humphries, 1985) (DT40, ATCC-CRL-2111). The online bursal transcript database established by the group of Dr. Jean-Marie Buerstedde (http://swallow.gsf.de/dt40.html) provides valuable information regarding genes involved in B-cell specific processes related to immunoglobulin repertoire formation, signal transduction, transcriptional control, apoptosis, and others.

The halogenated hydrocarbon 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is a widespread environmental pollutant. TCDD causes numerous biological effects that are mediated almost entirely through the activation of the aryl hydrocarbon receptor (AhR) (Schmidt and Bradfield, 1996). The characteristics of the receptor and its interactions were outlined above in Chapter I. Numerous reports have demonstrated the immunosuppressive effects of TCDD at the humoral and cellular level (Holsapple et al., 1991). The phenomenological effects of TCDD on the chicken bursa of Fabricius have been extensively reported. Various studies show that TCDD and its congeners cause inhibition of lymphoid development, bursal atrophy (Andersson et al., 1991), and decrease of bursal weight (Powell et al., 1996) in the developing chicken bursa of

Fabricius. It is suggested that the inhibition of lymphoid development in the bursa is caused by the direct effect of TCDD on the bursal microenvironment, resulting in a decrease in the attraction of stem cells and/or in the capacity to induce proliferation of the colonizing cells (Nikolaidis et al., 1990).

A complete study of the transcriptional profile of a chicken immune cell line upon exposure to TCDD has never been reported. The DT40 cell line has proven to be a reliable system with the potential to help complement understanding of the biochemical and physiological consequences of TCDD. The readily available chicken immune array created at the FHCRC (Neiman et al., 2001), includes a cDNA library with an average insert size of about 1.5 kb pairs prepared from DT-40 cell line (Abdrakhmanov et al., 2000), gene-identified clones from chicken activated T-cell cDNA library (Tirunagaru et al., 2000), and 50 selected non-overlapping expressed sequence tag (EST) clones (DKFZ426) from a bursal library developed from a normal 2-week old chicken at the German Resource Center (http://www.rzpd.de). This particular array represented about 2,200 different genes. The use of this array has allowed us to develop a comprehensive database of genes, affected directly or indirectly by TCDD, that could shed some light on the complex mechanism of toxicity induced by this chemical. In this study, we have used a variety of tools for exploring expression data, such as different clustering algorithms (Dysvik and Jonassen, 2001) and visualization methods (Sturn et al., 2002).

Our results reveal several clusters of interacting gene functions that may provide an explanation for the multiple effects of TCCD and serve as the basis to propose novel experimental hypotheses.

Materials and Methods

Cell culture

The DT40 B-cell line was purchased from American Type Culture Collection (ATCC, CRL-2111, Rockville, MD). The culture was propagated as suggested by ATCC, in Dulbecco's modified Eagle's medium with 2 mM L-glutamine and 0.05 mM 2-mercaptoethanol, 10% (v/v) tryptose phosphate broth, 10% (v/v) fetal bovine serum and 5% (v/v) chicken serum. Batches of DT40 cells were grown separately in 75-cm^2 flasks (Nunclon, 156499) and incubated at 37 ºC and 5% CO_2, until the cultures reached an approximate density of 2.3 X 10^6 cells per ml.

Chemical treatment and experimental design

For microarray analysis, cell suspensions were cultured for 72 h; the cells were counted and divided evenly into 75-cm^2 culture flasks. An initial number of approximately 1 x 10^5 cells per ml was used. The cells were incubated for an additional 48 h and then four flasks were exposed separately (two flasks per time point) to 1 nM and 10 nM of TCDD solubilized in dimethyl sulphoxide (DMSO), during 6 and 12 h. Control flasks were exposed to the same volume of DMSO. Cells were harvested from the TCDD-treated and the control flasks at 6 and 12 h post-exposure, respectively. Cells from each flask were processed individually for total RNA extraction.

For real-time PCR, cell suspensions were incubated for 72 h; the cells were counted and divided evenly into 6-well plates. An initial number of 1.3 x10^6 cells/well was used. Two plates were incubated for 48 h and then exposed to 1 nM of TCDD. The other two

were exposed to a similar volume of DMSO (controls). Cells were harvested from the TCDD-treated and the control plates at 1 and 6 h after exposure. Cells from each well were processed individually for total RNA extraction.

mRNA purification

mRNA was extracted using the Trizol® (Invitrogen, Life Technologies, 15596-026) reagent, precipitated with isopropanol, and further purified using oligotex mRNA spin-column protocol (Qiagen, 70042) to obtain poly-A^+ mRNA. Poly-A^+ mRNA was diluted in RNAse free water (Ambion, Austin, TX) and was quantified by UV absorbance at 260 nm.

Chicken immune cDNA microarray

A chicken immune cDNA microarray was constructed at the FHCRC (Neiman et al., 2001). The array included a cDNA library with an average insert size of about 1.5 kb pairs prepared from DT-40 cell line (Abdrakhmanov et al., 2000), gene-identified clones from chicken activated T-cell cDNA library (Tirunagaru et al., 2000), and 50 selected non-overlapping expressed sequence tag (EST) clones (DKFZ426) from a bursal library of a normal 2-week old bird developed at the German Resource Center (http://www.rzpd.de). The complete cDNA array contained approximately 4186 transcripts, representing about 2,200 different genes.

Sample preparation and hybridization

Labeling of cDNA samples, preparation of microarrays, and hybridization reactions were performed, as a custom service, by the FHCRC. Here, for informative purposes, the techniques are briefly described. The total extracted RNA was linearly amplified using a T7 promoter-based method (Ambion, Inc., Austin, TX) and a random prime labeling methodology. A minimum of 30 μg of each pool of total mRNA was used, and then transformed into cDNA. Target cDNAs from control RNA samples were labeled by reverse transcription using Cyanine-3 (Cy3, red) and the TCDD-treated samples were labeled with Cyanine-5 (Cy5, green). The coupling of both fluorophores was carried out using an established amino-allyl coupling procedure. The Cy3- and Cy5-labeled cDNA samples were combined and co-hybridized to the array for 16 hrs at 63°C, followed by a sequential, stringent washing procedure (Fazzio et al., 2001).

Image scanning and data processing

Hybridized slides were scanned using a GenePix 4000 Microarray scanner (Axon Instruments, Inc., Union City, CA). Two 16-bit tiff images were collected for each array corresponding to 532 nm (Cy3) and 635 nm (Cy5) excitation wavelengths. Image analysis was performed using GenePix Pro 4.0 software (Axon Instruments, Inc.). Normalization of Cy5 to Cy3 signal in each experiment was performed assuming equivalent global hybridization of test (TCDD exposed) and reference (control) probes to genomic chicken DNA.

Values of Log2 of the ratio of medians were filtered using the max/min criterium, which corresponded to one standard deviation above or below zero. The resulting values were processed using GENESIS (Institute for of Biomedical Engineering, Graz University of Technology).

Determining repression or induction

Fluorescent ratios on a microarray are optically analyzed to determine whether there has been repression or induction of any particular gene. The quantification of both fluorophores (red and green) is carried out by image scanning. In the present experiment green represented the control mRNA and red represented the experimental mRNA. A calculated ratio of red:green tells whether a particular gene was induced or repressed as a consequence of the experimental treatment in comparison to the baseline expression (of the non-treated cells) . Then, the expression ratio is calculated as a decimal value (e.g. 0.2 for a 5-fold reduction), and the decimal value is then transformed into logarithmic (base 2) scale, which makes it easier to visualize induction versus repression (Gasch et al., 2000).

$$\textit{Ratio: red : green} \quad \rightarrow \quad \textit{red} \div \textit{green} \quad \rightarrow \quad \log_2 (\textit{red} \div \textit{green})$$

If the expression of the experimental sample is greater than the control, the ratio will be greater than one, and then the final number after logarithmic transformation will be positive. The final number will be negative if the expression of the experimental sample is less than the control, and so if the ratio of the experimental sample and the control is 1:1, then the result will be zero. Thus, to easily assess the repression or induction, the

resulting data are converted into images using red and green as artificial colors. Because the same colors (red and green) have been used in the original hybridization experiment, this could be a source of confusion. It is very important not to forget that the artificial colors are just a mere representation of changes in expression and do not represent the initial fluorescence from the mRNA hybridization experiment. In a typical scale, a black spot on the array would indicate that equal amounts of red and green fluorescence were observed on the original spot, thereby giving an equal expression ratio of 1:1, and subsequently a logarithmic value of 0.

Clustering

The process of separating a set of objects, according to their similarity, into several subsets, is called clustering (Gilbert et al., 2000). In general, two strategies are possible: supervised (based on existing knowledge) and unsupervised clustering. In supervised clustering, existing biological information is used to guide the classification of genes affected by chemical treatment. Unsupervised clustering is used to define clusters that minimize intra-cluster variability while maximizing inter-cluster distances, i.e. finding clusters, whose members are similar to each other, but distant to members of other clusters in terms of gene expression based on the used similarity measurement.

Hierarchical clustering is referred to the method that identifies a small group of genes that share a common pattern of expression and then constructs the dendrogram in a sequential manner using a ranked series of clusters. Non-hierarchical clustering, such as k-means analysis, assigns each gene expression datum to a cluster based on its expression profile, and then repeats the process until every datum point has been placed in a cluster.

Results

Microarray analysis identified 114 clones that were either significantly down- or up-regulated at a certain dose or time point in response to TCDD exposure. The cut-off of the logarithmically transformed values used to consider a gene expression level significantly different between control and TCDD-exposed cells, was set two standard deviations above or below the average expression level of all genes represented on the array. Among the clones of potential interest, less than half of the corresponding genes were fully annotated in the public database (http://www.ncbi.nlm.nih.gov/), i.e., the function of the gene had not been described.

In order to obtain more information about these spots, the sequence of each of the clones would have to be compared (using BLAST searching) to the GeneBank or other dedicated databases (http://www.ncbi.nlm.nih.gov/;http://genome.ucsc.edu/; http://swallow.gsf.de/dt40.html).

This type of data mining has been performed for a random selection of 9 genes (see Table 2.1), but it could in theory be repeated for every one of the 114 clones of

interest. Table 2.1 shows a limited selection of genes, that points out some noteworthy analogies to what has been previously described in the literature in terms of TCDD intoxication mechanisms, including processes such as apoptosis, cell cycle regulation, respiratory chain (i.e. oxidative stress), and DNA damage. Regardless of whether their function has been described, hierarchical cluster analysis subdivided the 114 genes of interest into five clusters (Figs. 2.1, 2.2 and 2.3), based on similar behavior in the 4 different experimental treatments tested. The most typical behavior is displayed by the 18 genes in cluster "C", i.e., upregulation after 6 hours of exposure followed by downregulation after 12 hours of exposure. As shown in Fig. 2.3, the annotation of no less than 8 genes mentions "non-functional folate binding protein". Ten spots have no annotation whatsoever ("none"). As a consequence, only a limited portion of all listed links will actually be useful for further investigation.

Table 2.1. Selected genes from analysis of microarray of DT40 B-cells exposed to TCDD. Induced changes in expression involve apoptosis, metabolism, cell cycle regulation, DNA repair, and inflammation, among others.

Gene Bank Number	Gene name	Induced				Repressed				Function	Reference
		A	B	C	D	A	B	C	D		
1117827	α-mannosidase-II		1.1		0.4	-0.4		-2.1		Processing of N-linked glycans	(Misago et al., 1995)
107354	Leukocyte TIA1 precursor	3.0	0.7					-0.6	-1.6	Apoptosis in CTL targets	(Tian et al., 1991)
4455140	TNF-lig. B-cell stim (BLYS)	2.8					-0.7	-1.3	-0.2	Stimulator of B-lymphocytes	(Smith and Cancro, 2003)
1704889	cdc2/CDC28-like kinase		0.7	1.6	0.3	-1.6				Cell cycle regulation	(Johnson and Smith, 1991)
730163	NA-methyl-transferase				0.6	-1.8	-1.2	-0.8		Metabolism pyridines	(Aksoy et al., 1994)
6912497	Malonyl-CoA decarboxylase	0.4	0.4		1.2			-1.5		Metabolism Acetyl-CoA	(Sacksteder et al., 1999)
18105036	Cytochrome C oxidase	0.4	0.6	0.8	0.7					Respiratory chain	(Fabrizi et al., 1989)
603761	DNA repair protein RAD9		0.2	0.7		-0.4			-0.7	DNA damage checkpoint	(Volkmer and Karnitz, 1999)
120360	Matrix metallo-protease-2	0.3		0.3			-0.2		-0.9	ECM degradation, inflammation	(Goetzl et al., 1996)

Notes for treatments: A) 1 nM TCDD for 6 h; B) 1 nM TCDD for 12 h; C) 10 nM TCDD for 6 h; D) 10 nM TCDD for 12 h.

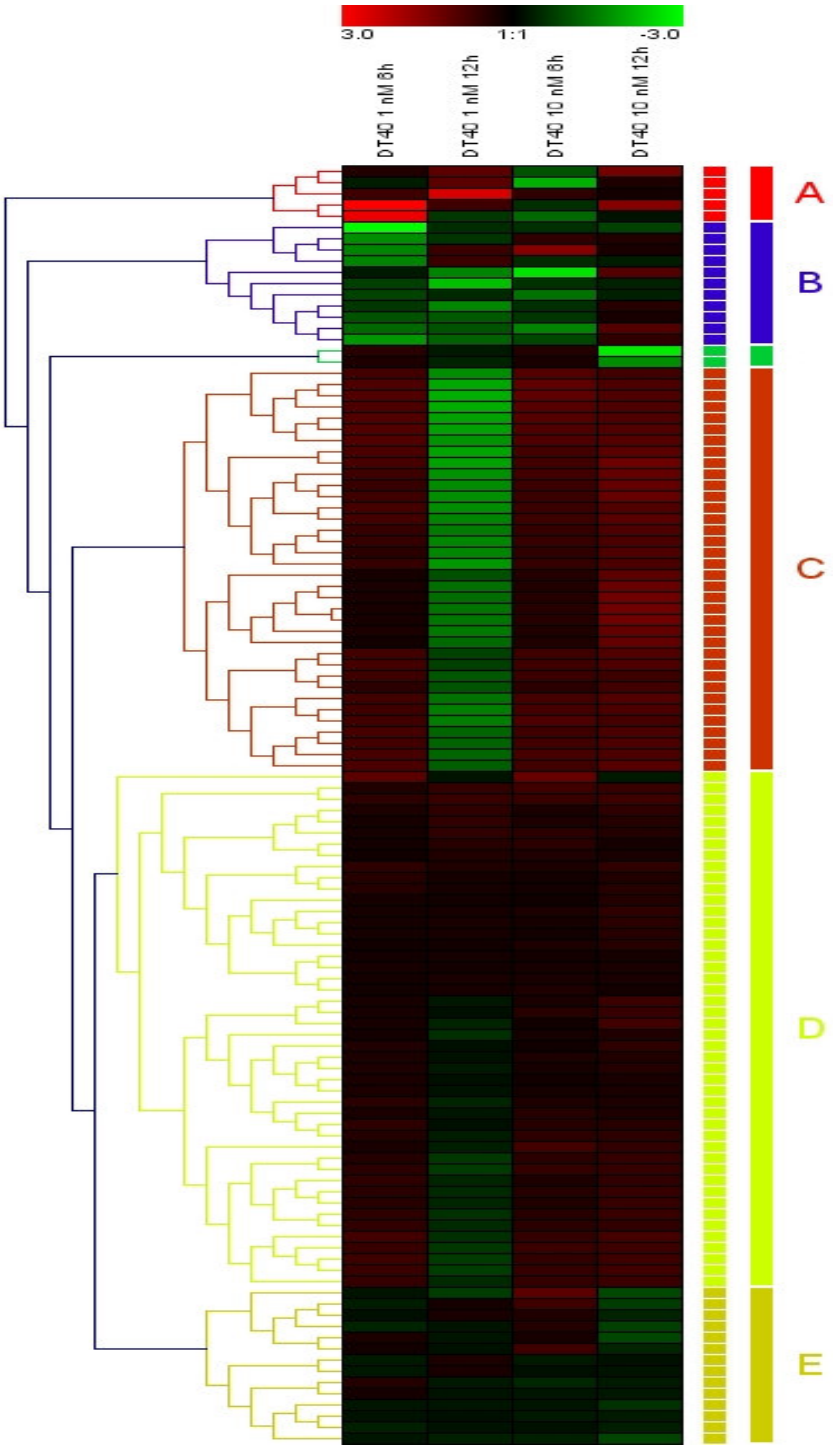

Figure 2.1. Cluster analysis of microarray data from DT40 cells exposed to TCDD: 1 nM of TCDD during 6 and 12 h (columns 1 and 2) and 10 nM during the same period of time (columns 3 and 4). The tree (dendrogram) is the result of a hierarchical clustering analysis using average linkage between genes. The analysis was performed using GENESIS microarray software (http://genome.tugraz.at/Software/GenesisCenter.html).

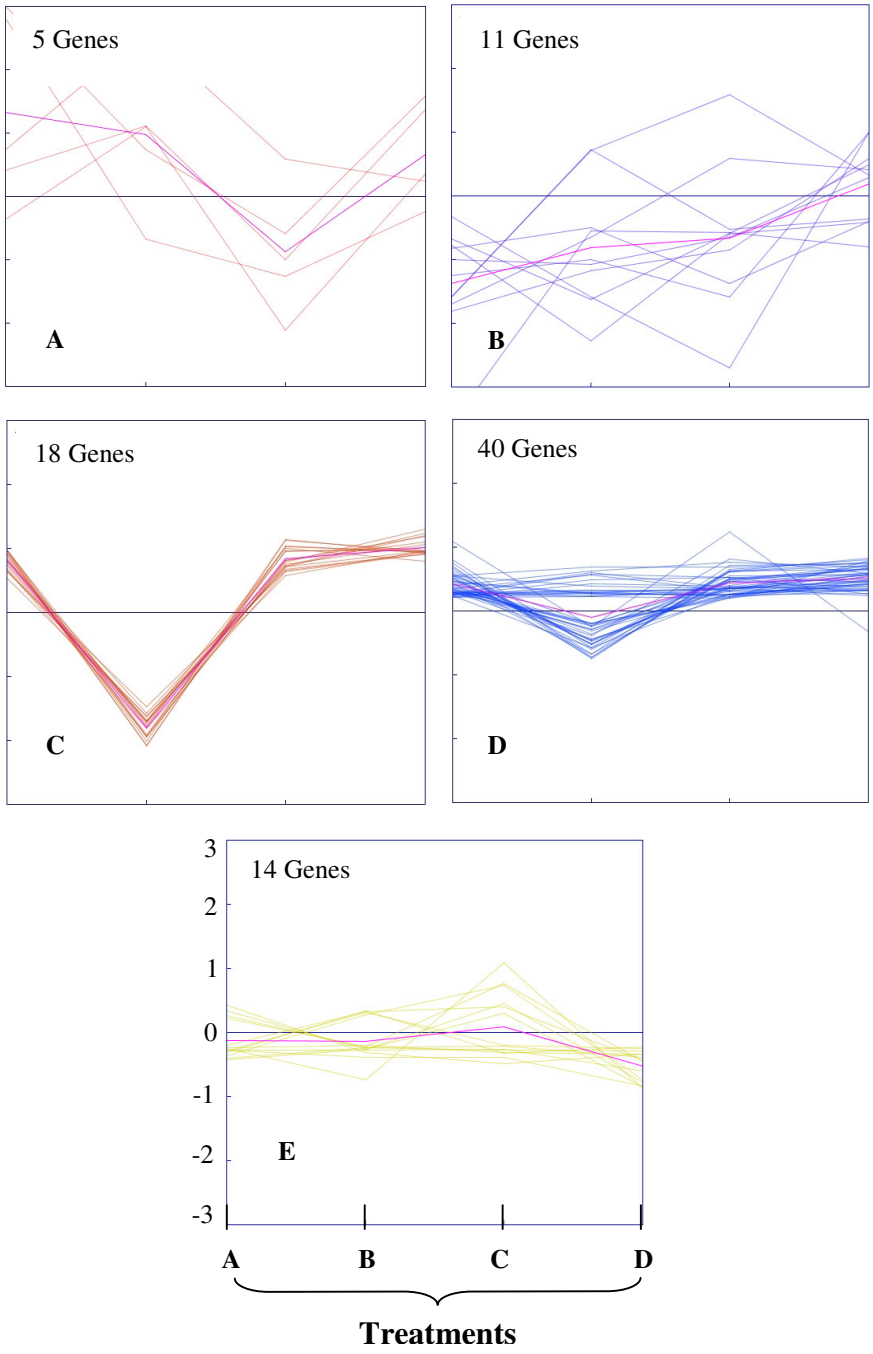

Figure 2.2. Gene expression profile using hierarchical clustering (HCL) in DT40 cells exposed to TCDD. The genes are grouped according to a common patter of expression, then the HCL algorithm constructs the dendrogram (Fig. 2.1) in a sequential manner using a ranked series of clusters (Schena, 2003). The positive numbers indicate the up-regulation of genes, and the negative numbers indicate down-regulation. The treatments were as follows: A: 1 nM for 6h; B: 1 nM for 12 h; C: 10 nM for 6 h; and D: 10 nM for 12 h.

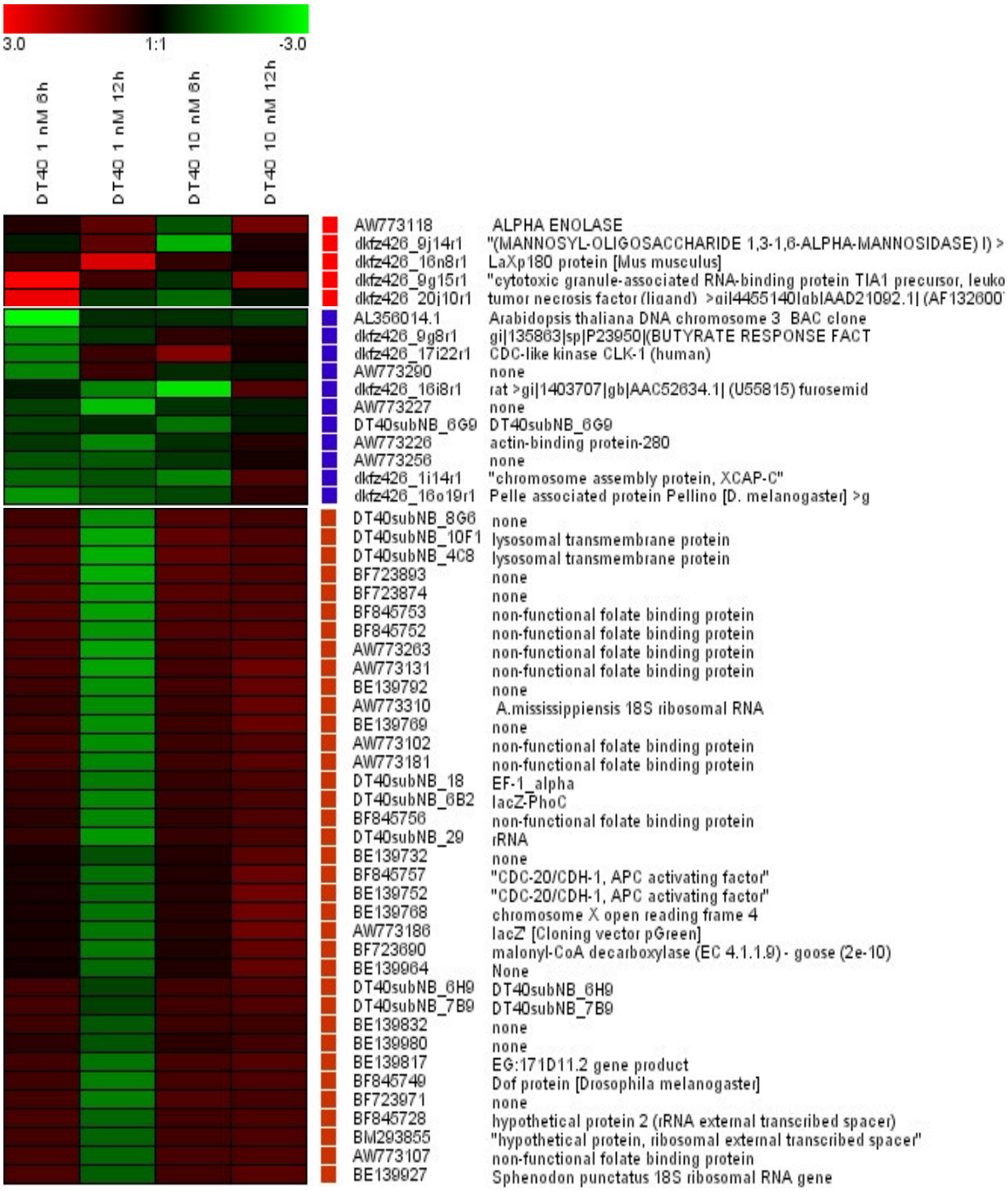

Figure 2.3. Clustering analysis of TCDD-exposed DT40 chicken B-cells. The genes were classified according to their average expression. This panel represents, starting from the top, clusters A, B and C shown above in Fig. 2.2. The four columns on the left show the 4 different treatment groups (see Fig. 2.2). The color-coded line to the left delineates the different gene clusters (A, B, C). The list of numbers shows the NCBI accession numbers as far as these are available and the last column shows a brief description for each gene, as far as gene function is known.

Discussion

TCDD has been demonstrated to suppress the humoral and cellular immune response, and to possibly increase the susceptibility of animals to several infectious diseases and other dysfunctions (Kerkvliet, 2002). The biological effects of TCDD have been correlated with its ability to bind and activate the aryl hydrocarbon receptor (AhR).

Following AhR activation, the transcriptional response of an impressive number of genes is altered, thus producing extensive toxic effects. B-cells are functionally compromised by direct exposure to TCDD (Holsapple et al., 1991; Sulentic et al., 1998; Sulentic et al., 2000). Some of the direct effects produced by TCDD in B-cells are, surprisingly, not dependent on expression of AhR (Davis and Safe, 1991). This suggests that at least some of the immunotoxicity of TCDD might be induced through different mechanisms. Recent investigations conducted by Kerkvliet and collaborators (2002) demonstrate, however, that both the lymphoid and myeloid cells (which originate from pluripotent hematopoietic cells in the bone marrow) represent critical AhR-dependent targets for TCDD's immunosuppressive effects. Irradiated $AhR^{-/-}$ mice reconstituted with $AhR^{+/+}$ bone marrow were highly immunosuppressed by exposure to TCDD, while those reconstituted with $AhR^{-/-}$ bone marrow were not. These data indicate that the AhR plays an essential role in the immunotoxicity of TCDD.

In this study, we have analyzed the transcriptional changes in DT40 cell line induced by TCDD exposure. The DT40 cell line maintains features related to its origin as an avian leucosis virus-induced B-cell tumor. Chicken B-cells develop a large repertoire of immunoglobulin genes by segmental gene conversion within the V-segment

(Weill and Reynaud, 1987). In birds, this occurs within the bursa of Fabricius. The strict compartmentalization of the B-cell development in the bursa of Fabricius of young chicks has been a powerful experimental advantage over other vertebrate species leading to numerous discoveries with regard to B-cell characterization, repertoire development, and tumorigenesis (Abdrakhmanov et al., 2000)

In previous preliminary investigations, we have observed the induction of genes that are considered key players in the apoptosis signaling pathway by exposing DT40 cells to 10 nM TCDD. These initial experiments were carried out using an EST collection containing approx. 27,000 clones from the chicken bursa of Fabricius that were spotted in duplicate onto two 21 x 21 cm nylon filters obtained through the German Genomic Resource Center (http://www.rzpd.de; results not shown). At the time we started this project (three years ago), this was the only publicly available tool for the functional genomic study of chicken B-cells. The list of upregulated genes obtained from these preliminary studies included: the tumor necrosis factor-receptor 1 (TNFR-1)-related caspase 8 and FADD (Fas-associated death domain). Other genes included the chicken cytochrome P450 (CYP1A4), glutathione-S-tranferase, and the transcription factor SRY. These results were used as the basis for further investigations, specifically with regard to the upregulated pro-apoptotic genes.

Given the difficulties experienced in the use of filter spotted arrays, especially the tedious and lengthy radioactivity-based hybridization procedures, as well as the lack of a dedicated software package for both image and data analysis, we decided to start using glass array technology. Using chicken immune glass cDNA arrays developed at

the Fred Hutchinson Cancer Research Center (Neiman et al., 2001) in a service-for-fee mode, improved reproducibility and cost-efficiency were attained and, more importantly, the earlier challenges regarding image and data analysis were resolved.

Unfortunately, the switch from radioactive phosphorus-based nylon membrane macroarrays to fluorescence-based glass microarrays did not take care of all the technical hurdles. The most important new challenge encountered with the chicken immune glass arrays, was the poor annotation that accompanied the collection of clones.

Even though most of the clones included in the array had been included in the GeneBank repository, a considerable number did not include any data regarding their sequence similarity to known genes. Nevertheless, from the clones that were selected by GENESIS as clones of interest (based on the fact that their expression level deviated more than two standard deviations from the average expression level of all spots on the array), a number of genes were related to apoptosis. As shown in Table 2.1, an important change was observed in the expression of leukocyte TIA1 precursor gene, known to be involved in the process of apoptosis of targeted cells by cytotoxic lymphocyte (CTL) (Tian et al., 1991). We also observed induction of the TNF-ligand B-lymphocyte stimulator (BLYS) (Smith and Cancro, 2003) and of cytochrome C oxidase (Fabrizi et al., 1989). Finally, upregulation of DNA repair protein RAD9, a protein that is phosphorylated in response to DNA damage (Volkmer and Karnitz, 1999) pointed in the same direction.

This, in combination with similar information obtained from the above mentioned preliminary filter array analyses, led us to pursue (see Chapter III) the working hypothesis that exposure to TCDD induces a shift in various apoptosis-related genes that would ultimately lead to loss of pre-B-cells in the bursa of Fabricius (Puebla-Osorio et al., 2004). If valid, this assumption could help explain the weight loss and atrophy of the bursa of Fabricius in earlier reports (Andersson et al., 1991; Nikolaidis et al., 1990).

Although the time frame of this study did not allow us to pursue more than one working hypothesis distilled from the cDNA microarray technology, and despite the limitations of this particular microarray, it is quite clear that further data mining of the clones that responded to TCDD exposure has the potential to generate additional working hypotheses and thus warrants further investigation.

CHAPTER III

2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN (TCDD) ELICITS ARYL HYDROCARBON RECEPTOR (AHR) - MEDIATED APOPTOSIS IN THE AVIAN DT40 PRE-B-CELL LINE THROUGH ACTIVATION OF CASPASES 9 AND 3*

Overview

The halogenated aromatic hydrocarbon 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is known to induce immunotoxicity, but relatively little is known regarding its effects on B-lymphocytes, and on avian B-cells in particular. In this study, the avian bursal pre B-cell line DT40 was exposed to TCDD ranging from 1 to 500 nM for 1 and 6 hours. At 100 nM, TCDD caused a significant increase in the number of apoptotic cells, as assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) assay, and induced the expression of the chicken cytochrome P450 1A4 (CYP1A4) mRNA, a hallmark of TCDD exposure. TCDD induced transient upregulation of AhR mRNA. At 100 nM, both caspase 3 and caspase 9 were transiently upregulated after 1 hour, but returned to normal levels after 6 hours of exposure.

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*Reprinted from Comparative Biochemistry and Physiology Part C, Puebla-Osorio N., Ramos K.S., Falahatpisheh M.H., Smith R. III and Berghman L.R., "2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) elicits aryl hydrocarbon receptor (AhR) - mediated apoptosis in the avian DT40 pre-B-cell line through activation of caspases 9 and 3", 2004, with permission from Elsevier.

Challenge with TCDD after AhR blockade with resveratrol, a competitive AhR antagonist, prevented changes in caspases 3 and 9 and in the AhR message itself, suggesting that the effects of TCDD were mediated via the AhR. TCDD did not cause significant changes in the relative gene expression of caspase 8, Bcl-2 and Bcl-xL. We conclude that avian DT40 pre B-cells exposed to TCDD are susceptible to apoptosis, likely through activation of executioner caspase 3.

Introduction

2,3,7,8-tetra-chlorodibenzo-p-dioxin (TCDD) is a potent contaminant that belongs to a large group of halogenated aromatic hydrocarbons. The halogenated aromatic hydrocarbons are ubiquitous and persistent environmental toxicants. It is believed that most biological effects of this group of compounds are mediated by the activation of the aryl hydrocarbon receptor (AhR) (Schmidt and Bradfield, 1996). The AhR is a ligand-activated transcription factor, member of the basic helix-loop-helix (bHLH) and PAS (Per/Arnt/Sim) domain family of proteins (Whitlock, 1999). Upon hydrocarbon ligand binding, the AhR dissociates in the cytosol from two 90 kD heat shock proteins and the Hepatitis virus X-associated protein 2 (XAP2). The AhR then translocates to the nucleus and dimerizes with the aryl hydrocarbon receptor nuclear translocator (Arnt) (Carver and Bradfield, 1997). The AhR/Arnt heterodimer interacts with dioxin responsive elements (DRE) located in the regulatory region of target genes resulting in their activation or repression. These DRE-containing genes include metabolic enzymes (Nebert, 1994), growth factors (Kim et al., 2000; Zaher et al., 1998)

and cytokines (Lai et al., 1996). Consequently, AhR has been associated with a wide diversity of disorders, including immunosuppression, thymic atrophy, cleft palate, atherosclerosis, chloracne, reproductive dysfunction and certain types of cancer (Kim et al., 2000; Puga et al., 2000; Zaher et al., 1998). Puga et al. (2000) have studied the transcriptional profile of human HepG2 cells exposed to TCDD, showing alteration of the expression of more than 300 known genes by a factor of at least 2.1. Those genes were classified according to their functions, which included regulation of cell cycle, differentiation, apoptosis, development, cell adhesion, cancer, and metastasis, and genes coding for transcription factors.

Previous studies have demonstrated the disruptive effects of TCDD on humoral and cellular immunity (Holsapple et al., 1991). In mammals, TCDD suppresses humoral immunity by inhibiting IgM secretion by B-lymphocytes during differentiation, with only modest effects on B-cell proliferation (Dooley and Holsapple, 1988; Luster et al., 1988; Wood and Holsapple, 1993). The ability of TCDD to suppress the IgM secretion is associated with the presence of a DRE within the Ig heavy chain 3' β enhancer in the CH12.LX B cell line (Sulentic et al., 1998; Sulentic et al., 2000). While immunosuppression by TCDD is believed to be primarily mediated through the AhR, other studies have suggested that TCDD is capable of inducing apoptosis in T-cell lines in an AhR-independent process following upregulation pro-apoptotic genes (Hossain et al., 1998). TCDD has also been shown to induce apoptosis in AhR-deficient El-4 mouse thymoma cells (Park et al., 2003).

In birds, little is known about the immune effects of TCDD. Peden-Adams et al. (1998) have reported dose-dependent suppression of B-cell proliferation in hatchlings from TCDD-injected eggs, and Fox and Grasman (1999) described reduction of both bursa of Fabricius and thymus weights after in ovo administration of TCDD and 3,3',4,4',5-pentachlorobiphenyl (PCB126). The underlying gene interactions that mediate these phenomena have not been investigated to date.

In the present study, we analyzed the mRNA expression of chicken caspases 3, 8 and 9, the anti-apoptotic genes Bcl-2 and Bcl-xL, and ligand-related genes including AhR, Arnt, and CYP1A4 in DT40 B-cells. Blockage of the AhR with the competitive antagonist resveratrol (3,5,4'-trihydroxystilbene) was used to identify the role of the AhR in the gene shifts caused by TCDD exposure.

Materials and Methods

Cell culture

The DT40 B-cell line is an avian leucosis virus (ALV)-induced bursal lymphoma cell line derived from a Hyline SC chicken (ATCC, CRL-2111, Rockville, MD). Cell cultures were propagated in Dulbecco's modified Eagle's medium (DMEM) with 2 mM L-glutamine and 0.05 mM 2-mercaptoethanol, 75% (v/v); 10% (v/v) tryptose phosphate broth; 10% (v/v) fetal bovine serum; and 5% (v/v) chicken serum, and cultured at 37 ºC in a 5% CO_2-balanced air environment in a 75 cm^2 culture flask.

Chemical treatments and experimental design

DT40 cell suspensions were incubated for 48 h; counted and plated in 6-well plates at a density of approximately 1 x 10^6 cells per well. Plates were incubated for an additional 24 h and then exposed to 1, 10 or 100 nM of TCDD solubilized in dimethyl sulphoxide (DMSO) or the same volume of DMSO for 1 and 6 h. At the end of treatments, cells were harvested and processed individually for total RNA extraction. To assess whether the changes observed in the studied genes were AhR related, DT40 cells were incubated for 1 h, prior to exposure to TCDD, with 5 μM of resveratrol (Calbiochem, San Diego, CA), a competitive AhR inhibitor.

CYP1A4 induction

Upregulation of Cytochrome P450 (CYP), including CYP1A4 in the chicken is considered a hallmark of TCDD responsiveness (Walker et al., 2000). To prevent constitutive repression of CYP1A4 transcription and ensure hydrocarbon inducibility, protein synthesis was blocked by pre-treatment of DT40 cells with cycloheximide (Sigma, St. Louis, MO) at 10 μg/ml during 6 hours as described (Ou and Ramos, 1995). The cells were then exposed to 10 nM of TCDD or an equal volume of DMSO for 1 and 6 h, respectively.

TUNEL assay

To study fragmentation of genomic DNA in apoptotic cells, DNA fragments representing numerous 3'-hydroxyl termini were labeled with fluorescently-tagged

deoxyuridine triphosphate nucleotides (F-dUTP) (Overbeeke et al., 1998). In this assay, the enzyme Terminal Deoxynucleotidyl Transferase (TdT) catalyzes a template-independent addition of deoxyribonucleoside triphosphates to the 3'-hydroxyl ends of double- or single-stranded DNA.

TUNEL was performed using the APO-DIRECT kit (Phoenix Flow Systems, San Diego, CA) after the cells were exposed to 0.5 μM, 100 and 10 nM TCDD, and DMSO for 1 and 6 h. Cells exposed to 10 μM H_20_2 for 20 minutes were used as a positive control. F-dUTP incorporation was analyzed by flow cytometry (FACSCalibur, Becton Dickinson).

RNA extraction and cDNA synthesis

Total RNA was extracted from each group of cells using Trizol® (Invitrogen, Carlsbad, CA). Total RNA concentrations were determined by OD_{260}. Poly-A^+ RNA was purified using Oligotex® (Qiagen, Valencia, CA).

cDNA synthesis was carried out using the 2-Step RT-PCR method (RETROscript Protocol; Ambion, Austin, TX). Briefly, 100 ng of Poly-A^+ mRNA was combined with 1 μl of oligo dT (50 μM) and denatured at 70 ºC for 10 min. Then, 1 μl of RNAse inhibitor (10 U/μl), 2 μl 10X RT buffer, 4 μl of dNTP mix (2.5 μM each dNTP final concentration), and 1 μl of MMLV reverse transcriptase (100 U/μl) were added to a final volume of 20 μl. The mix was incubated at 42 ºC for 60 min. The resulting cDNA was denatured at 92 ºC for 10 min and stored at –80 ºC.

Real-Time PCR (Quantitative RT-PCR)

Real-time PCR amplification was performed using the ABI Prism 7900 Sequence Detection System (PE-Applied Biosystems), in 384- or 96- well plates. Primers used for the PCR (Table 3.1) were designed using Primer Express 4.1 (ABI systems, Applied Biosystems) and the GeneFischer on-line source (http://bibiserv.techfak.uni-bielefeld.de/genefisher/). The target genes included is this study were: chicken caspase 3, caspase 8, caspase 9, Bcl-2, Bcl-xL, AhR, Arnt, cytochrome P450 (CYP1A4), and β-actin as endogenous control. Each reaction included 0.5 µg of cDNA sample, 12 µl of SYBR Green master mix (PE-Applied Biosystems) and 1 µM of the appropriate gene-specific forward and reverse primers, in a final reaction volume of 20 µl. The thermal cycling conditions included an initial denaturing step at 95°C for 10 min, 50 cycles at 95°C for 15 s, and 65°C for 1 min. All experiments were performed in triplicate for each data point. Each of the averages shown in the Figures is the result of three samples, each run in duplicate, totaling six samples per group.

To verify the amplicon size of all target genes, the products from the real-time PCR were run on a NuSieve 3:1 (Cambrex Bio Science, Rockland, ME) agarose gel and visualized with ethidium bromide. The effect on the relative gene expression that resulted from TCDD-exposure of DT40 cells was calculated using the $\Delta\Delta C_T$ method (Lehmann and Kreipe, 2001; Livak and Schmittgen, 2001), using the following formula:

$$\frac{mRNA \text{ levels in exposed}}{mRNA \text{ levels in control}} = 2^{-\Delta\Delta C_T}$$

where: $\Delta\Delta C_T = (C_T^{\text{target gene}} - C_T^{\beta\text{-actin}})_{\text{exposed}} - (C_T^{\text{target gene}} - C_T^{\beta\text{-actin}})_{\text{control}}$

The amplification efficiency for each of the tested genes was determined using β-actin as an internal control. Each reaction included 0.5 μg of cDNA sample per gene, and 4 dilutions (1:1, 1:10, 1:100, and 1:1000); each dilution was run in duplicate (Muller et al., 2002).

Statistical analysis

The average $2^{-\Delta\Delta CT}$ values per treatment included six observations per group at each time point. Analysis of variance was performed, and statistical differences between means were calculated using LSM ($p<0.05$) (SAS Institute, Carey, NC, USA).

Results

Validation of the DT40 model

TCDD concentrations and exposure times were based on the literature (Matikainen et al., 2001; Tian et al., 1999) and on preliminary data (results not shown), showing that exposures in the range 10 - 100 nM of TCDD for 1 to 6 hours were optimal for early TCDD-induced gene expression alterations in DT40 cells.

For relative quantification of target genes, triplicate cDNAs from each time point following exposure to TCDD, were amplified using primers for the chicken caspase 3, caspase 8, caspase 9, AhR, Arnt, CYP1A4, Bcl-2 and Bcl-x_L, and β-actin. Since SYBR Green indistinctly binds to double-stranded DNA and other products may be detected along with the target gene, the amplification of a single target was verified for each sample by running a heat dissociation protocol after the final cycle of the PCR (Morrison

et al., 1998; Ririe et al., 1997). The heat dissociation curve for each particular target consistently produced a single dissociation peak for each of the target genes and for the reference gene (data not shown). The expected amplicon size and the presence of a single product per target gene, after a final dissociation protocol, were confirmed by NuSieve gel electrophoresis (Fig. 3.1). The amplification efficiency was calculated for all genes, the values for β-actin, caspase 3, caspase 9, Bcl-2, AhR, Arnt, and CYP1A4 ranged between 2.5 and 2.3, with $R^2 > 0.95$, fulfilling the criteria suggested by (Muller et al., 2002).

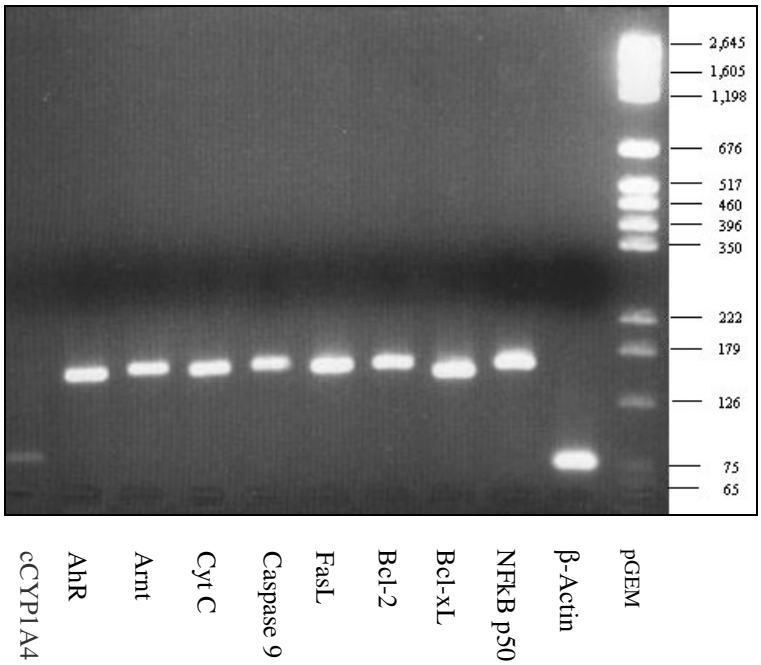

Figure 3.1. Electrophoretic verification of amplicon size obtained by real-time PCR on a NuSieve agarose gel. Visualization was done with ethidium bromide. Each of the reaction products was obtained after running dissociation curves in an ABI 7900.

The extent to which TCDD was capable of inducing apoptosis in avian B-cells was assessed by TUNEL assay . The percentage of apoptotic cells in cultures exposed to 500 and 100 nM of TCDD for 6 hours, respectively, was more than twice the percentage caused by administration of the vehicle (DMSO), i.e. 5.5 ± 0.18 %, as opposed to 2 ± 0.01 % in the control group. No significant increase in apoptosis was observed in cells exposed to 10 nM of TCDD (Table 3.2).

Table 3.1. Real-time PCR primers.

Gene Name	Gene Bank Number	Forward	Reverse	Expected Amplicon Size
cCaspase 8	AY057939	5'ACTGACATGGACTGAAGGA3'	5'TTACAGTGACGTGCTCCA3'	150
cCaspase 9	AY057940	5'TCAGACATCGTATCCTCCA3'	5'AAGTCACAGCAGGGACA3'	150
cCaspase 3	AF083029	5'GATGCTGCAAGTGTCAGA3'	5'ATCGCCATGGCTTAGCA3'	145
cBcl-xL	U26645	5'GCTTTGAGCAGGTAGTGA3'	5'CAAGTACGTGGTCATCCAA3'	147
cBcl-2	AY186728	5'TGAGCAGAGGTCACGTA3'	5'CACACTGTGGAACAGCA3'	150
cβ-actin	L08165	5'CTGATGGTCAGGTCATCACCATT3'	5'TACCCAAGAAAGATGGCTGGAA3'	100
cAhR	AF192502	5'ACACCTACTGGCTGTGA3'	5'GACAGTCATTCCACTCTCA3'	146
cARNT	AF348088	5'ACAGGAACTTCTGGGGAA3'	5'GAGCTGGTTCTCATCCA3'	148
cCYP1A4	X99453	5'CCTGTCCTGGTGCATGATGTAC3'	5'TCTGATCCAGCTCTGCCTGAA3'	74

The primers were designed from chicken gene sequences. Gene access numbers can be verified at www.ncbi.nlm.nih.gov/. Each primer sequence was verified for uniqueness by comparison with genomic sequences using BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Table 3.2. Percentage of apoptotic cells assessed by TUNEL assay.

Concentration (nM)	DMSO	H_2O_2	10	100	500
F-UTP labeled cells (%)	2.0±0.01	66.7±0.1*	2.5±0.01	4.2±0.14*	5.5±0.18*

Values are mean percentage change ± S.E. (n=2). *P<0.05. Quantitation of apoptosis was assessed by TUNEL assay. Percent of incorporation of dUTP in DT40 cells exposed for 6 h to TCDD. Significant differences were observed in groups exposed to 500 and 100 nM of TCDD.

Cytochrome P450 (CYP1A4)

Upregulation of cytochrome P450 (CYP1A4), a gene involved in cellular detoxification, was evaluated. In the chicken, the 1A4 isoform has been reported to be highly inducible in a wide variety of tissues by TCDD (Walker et al., 2000).

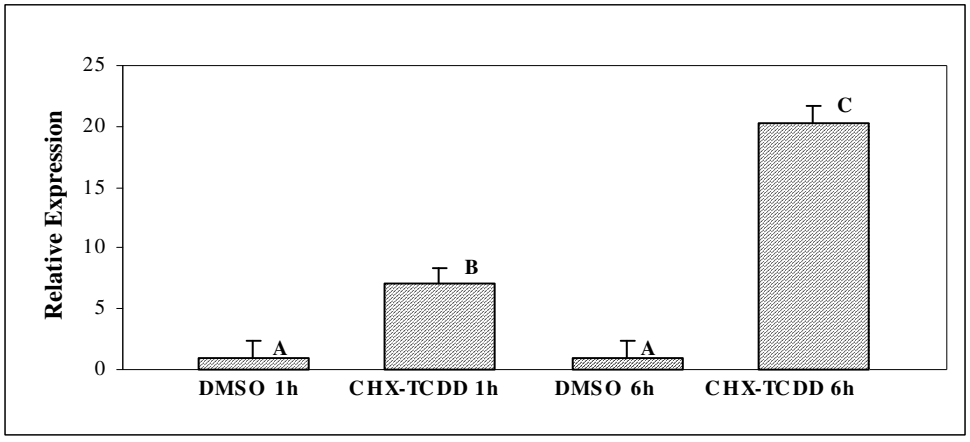

Figure 3.2A. Effect of CHX-TCDD on the expression of CYP1A4 in DT40 cells. Relative expression of the CYP1A4 gene using β-actin as calibrator gene. DT40 cells were pre-treated with the protein synthesis inhibitor cycloheximide (CHX, 10 μg/ml) for 6 h, and then exposed to 10 nM TCDD. Each column represents the average of six ΔΔCT values obtained by real time PCR, analyzed in duplicate. The bars represent the means ± standard error. The columns with same letters are not significantly different (p<0.05).

Initial attempts to induce CYP1A4 gene expression by exposing DT40 cells to different doses of TCDD were unsuccessful, likely due to repression of transcriptional activation by a labile repressor protein, as reported previously for mesenchymal cells (Ou and Ramos, 1995).

This was confirmed in experiments showing that blocking of protein synthesis by pretreatment with cycloheximide (10 μg/ml for 6 hours), followed by exposure to TCCD for 1 and 6 hours, caused a dramatic increase (up to 20-fold) in the expression of CYP1A4 (Fig. 3.2A).

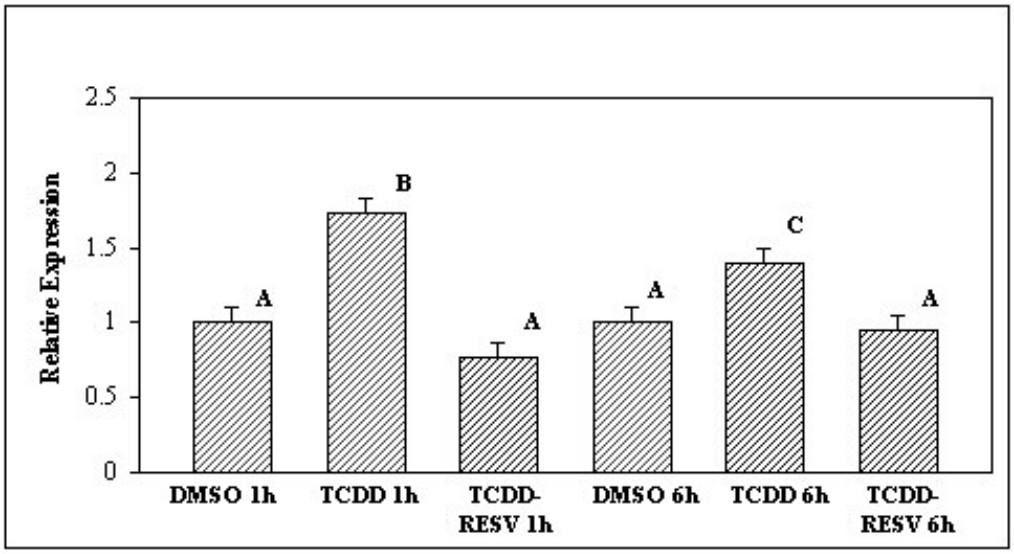

Figure 3.2B. Effect of resveratrol-TCDD on the expression of CYP1A4 in DT40 cells. Relative expression of the CYP1A4 gene. The cells were pre-treated with the AhR inhibitor resveratrol (5μM) for 1h, and then exposed to 100 nM TCDD. Each column represents the average of four ΔΔCT values obtained by real time PCR, analyzed in duplicate. The bars represent the means ± standard error. The columns with same letters are not significantly different ($p<0.05$).

The finding that TCDD induced apoptosis and activated P450 expression in our avian B-cell model warranted further analysis of underlying signal transduction pathways.

We attempted to demonstrate the inhibitory effects of resveratrol on CYP1A4 expression, and considering the unsuccessful efforts in inducing CYP1A4 expression at low concentration of TCDD, we pre-incubated the cells with 5 μM of resveratrol prior to exposure to 100 nM of TCDD and without exposing the cells to cyclohexamide.

Our results indicate a discrete but significant increase in gene expression of CYP1A4 after 1 h [1.72 ± 0.1 (SD) vs. 1.0; $P<0.05$] and 6 h [1.4 ± 0.1 (SD) vs. 1.0; $P<0.05$] of exposure to TCDD (Fig. 3.2B).

These results are in contrast with the groups pre-incubated with resveratrol for 1 h and then exposed to TCDD for 1 h [0.77 ± 0.1 (SD) vs. 1.0; $P<0.05$] and 6 h [0.96 ± 0.1 (SD) vs. 1.0; $P<0.05$] (Fig 3.2B). This is clear evidence that the effects of TCDD on CYP1A4 are AhR related.

AhR and Arnt

At the most distal point of the signal transduction pathway, it appeared that exposure to 10 nM TCDD induced a transient upregulation of AhR mRNA. After 1 hour of TCDD exposure, a 3.5-fold increase in AhR mRNA was observed; this effect completely disappeared after 6 hours of TCDD treatment (Fig. 3.3A). The same effect was observed after a 100 nM TCDD treatment, but the upregulation after 1 hour, though

significant, was only 50 % (Fig. 3.3B). A small but significant increase of Arnt mRNA was observed after 6 h of 10 nM TCDD induction (Fig. 3.4).

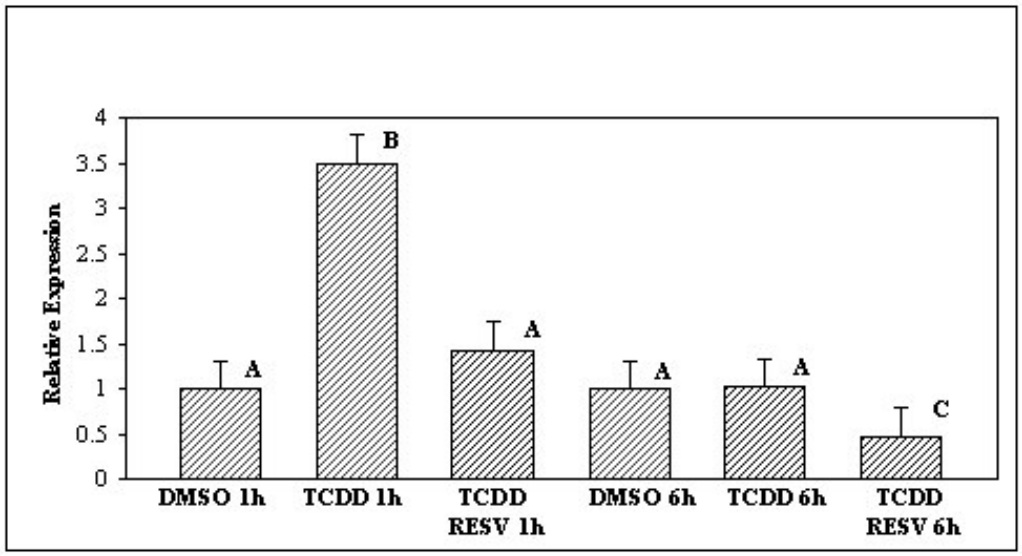

Figure 3.3A. Effect of TCDD on the expression of AhR in DT40 cells. Relative expression of AhR after 1 and 6 h exposure to 10 nM TCDD, with or without pretreatment with resveratrol.

Pretreatment of the cells with resveratrol (3,5,4'-trihydroxystilbene) a naturally occurring phytoalexin present in grapes and other foods that is a competitive antagonist of the AhR (Amakura et al., 2003), was used to assess the involvement of AhR in TCDD-induced gene expression alterations.

The inhibitory effect of resveratrol on AhR has been observed in human mammary epithelial cells (Chen et al., 2004), hepatocytes (Andrieux et al., 2004) and HepG2 cells (Ciolino et al., 1998). As expected, resveratrol completely blocked the upregulation of AhR mRNA induced by a 1 h TCDD exposure. After 6 hours, co-

administration of resveratrol and TCDD had even significantly decreased AhR mRNA as compared to vehicle. Resveratrol also blocked the effects of a 100 nM TCDD exposure on AhR mRNA induction (Fig.3.3B).

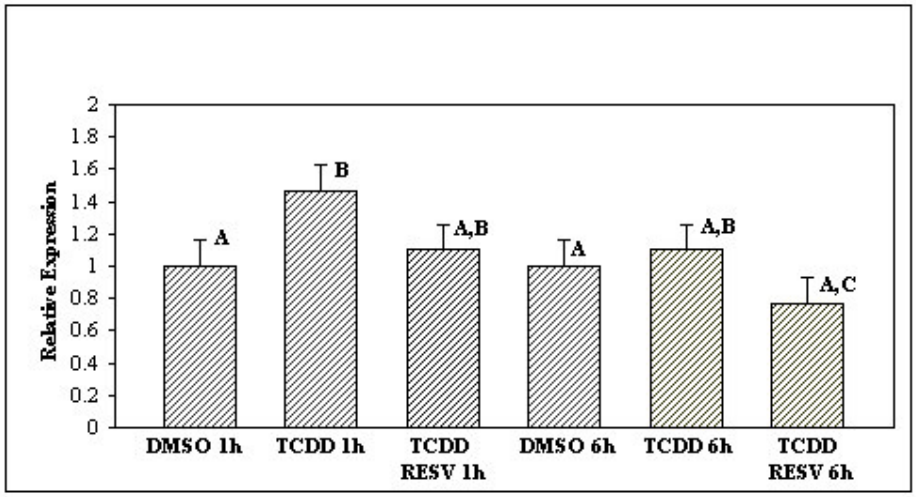

Figure 3.3B. Effect of TCDD (100 nM) on the relative expression of AhR in DT40 cells after 1 and 6 h of exposure with and without pretreatment with resveratrol. Each column represents the average (±SE) of ΔΔCT values obtained by real-time PCR correspondent to three samples in duplicate using β-actin as the calibrator. The bars represent the means ± standard error. The columns with same letters are not significantly different ($p<0.05$).

Apoptosis-related genes

Caspases are interconnected in a complex regulatory cascade that will ultimately result in the activation of DNA fragmentation machinery. Within this cascade, caspase 9 is located upstream, is usually activated by intrinsic or extrinsic death signals, and is therefore considered an initiator caspase.

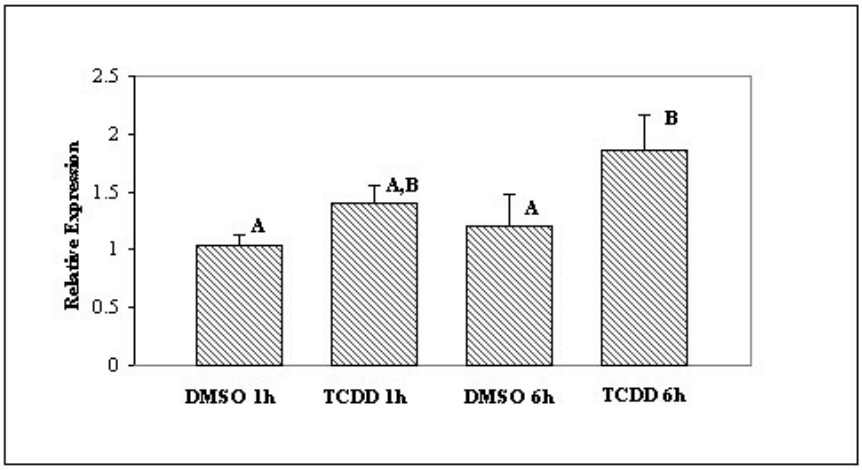

Figure 3.4. Effect of TCDD on the relative expression of Arnt in DT40 cells after 1 and 6 h of exposure. Each column represents the average (±SE) of six ΔΔCT values obtained by real-time PCR, correspondent to three samples analyzed in duplicate. The bars represent the means ± standard error. The columns with same letters are not significantly different ($p<0.05$).

Caspase 3, on the other hand, is located downstream of the cascade; it is an executioner caspase that will catalyze the activation of caspase-activated DNases such as CAD. The mean relative expression of caspase 9 was two times higher in the 100 nM TCDD treated group after 1 h compared to the DMSO group [2.9 ± 0.4 (SD) vs. 1.0; $P<0.05$] (Fig. 3.5A). This increase was no longer statistically significant after 6 h of exposure. At the 10 nM TCDD dose (Fig. 3.5B), the mean relative expression of caspase 9 mRNA was almost two times higher after 1 h compared to the control group [1.8 ± 0.15 (SD) vs. 1.0; $P<0.05$]. This induction in gene expression was entirely prevented in cells pretreated with resveratrol and then exposed to TCDD for 1 h [1.8 ± 0.12 (SD) TCDD vs. 1.20 ± 0.1 resveratrol; $P<0.05$].

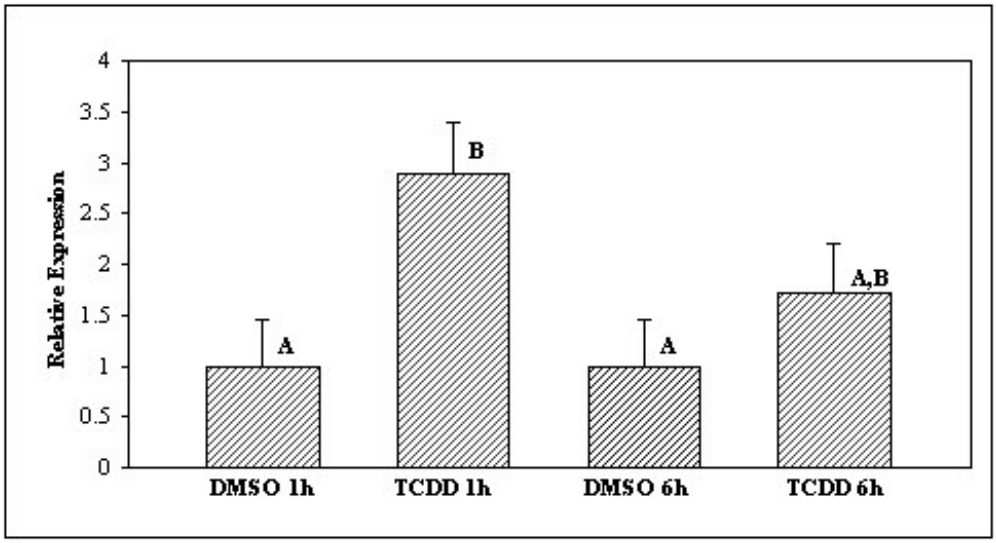

Figure 3.5A. Effect of TCDD (100 nM) on the expression of caspase 9 in DT40 cells after 1 and 6 h of exposure without pretreatment with resveratrol. Each column represents the average (± standard error) of six ΔΔCT values obtained by real-time PCR, correspondent to three samples analyzed in duplicate using β-actin as the calibrator. The columns with same letters are not significantly different ($p<0.05$).

After 6 h of TCDD treatment, the mean of expression of caspase 9 was 4-fold lower in the TCDD-exposed group, than in the control group [0.25 ± 0.12 (SD) TCDD vs. 1.0 ± 0.1 DMSO; $P<0.05$]. This trend was reversed by pretreatment with resveratrol. The mean relative expression of mRNA expression (normalized against the expression of β-actin) of caspase 3 (Fig. 3.6A) was almost two-fold higher in the group exposed for 1 h to 100 nM of TCDD, compared to the control (DMSO) group [1.9 ± 0.12 (SD) vs. 1.0; $P<0.05$]. This induction in gene expression was entirely prevented by pretreatment with resveratrol [1.9 ± 0.12 (SD) TCDD vs. 1.04 ±0.30 resveratrol + TCDD; $P<0.05$].

No difference was observed, however, between the TCDD exposed and the control group after 6 h of exposure.

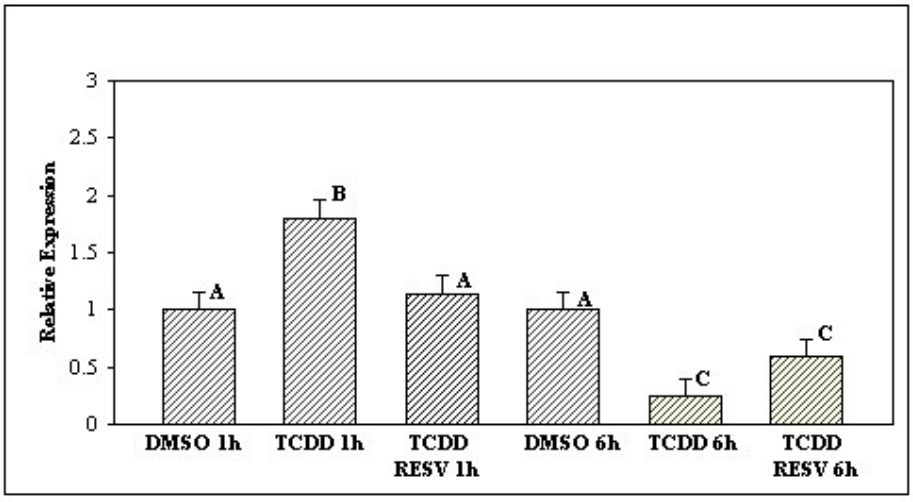

Figure 3.5B. Effect of TCDD (10 nM) on the expression of caspase 9 in DT40 cells after 1 and 6 h of exposure with and without pretreatment with resveratrol. Each column represents the average (± standard error) of six ΔΔCT values obtained real time PCR correspondent to three samples in duplicate using β-actin as the calibrator. The columns with same letters are not significantly different ($p<0.05$).

The mean relative expression was significantly lower in the resveratrol-pretreated group than in the TCDD-exposed group [1.2 TCDD ± 0.3 (SD) vs. 0.68 resveratrol ± 0.05; $P<0.05$]. At 10 nM TCDD and 1 h of exposure, however, the mean expression of caspase 3 was two-fold lower in the TCDD-exposed group than both the control (DMSO) and the group pretreated with resveratrol [0.68 ± 0.04 (SD) TCDD vs. 0.44 ± 0.04 resveratrol; $P<0.05$] (Fig. 3.6B).

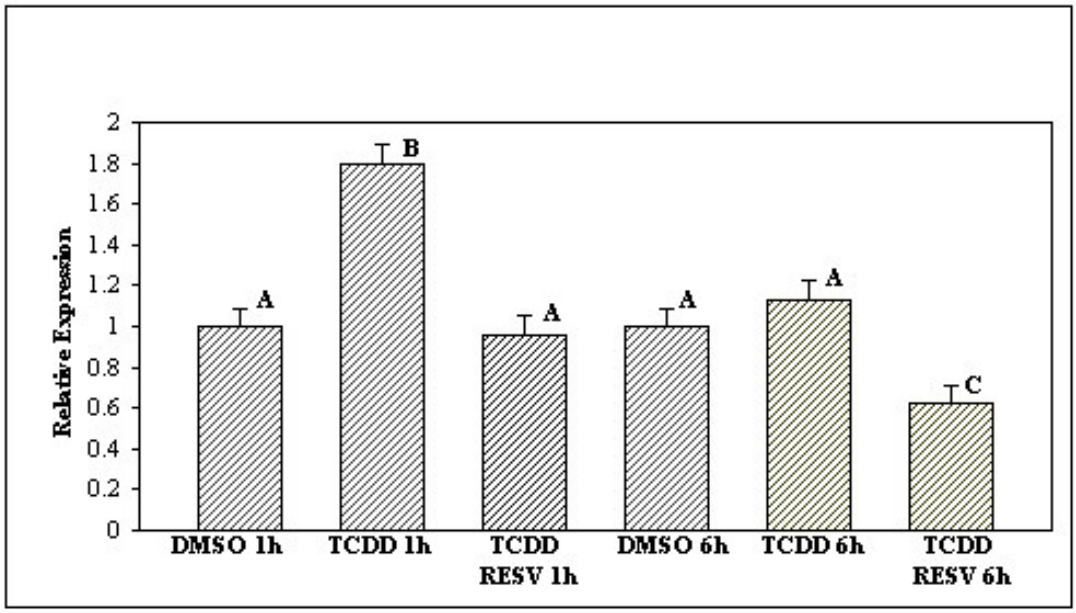

Figure 3.6A. Effect of TCDD (100 nM) on the expression of caspase 3 in DT40 cells after 1 and 6 h exposure with and without pretreatment with resveratrol. Each column represents the average (± standard error) of six ΔΔCT values obtained by real-time PCR, correspondent to three samples analyzed in duplicate using β-actin as the calibrator. The columns with same letters are not significantly different ($p<0.05$).

The same trends were observed after a 6-hour exposure to 10 nM of TCDD, although the TCDD-induced decrease of caspase 3 mRNA was no longer significant. No effects of TCDD exposure on the relative expression of caspase 8 could be seen under the conditions used in this study (results not shown). The involvement of Bcl-2 family Bcl-xL in TCDD-induced apoptosis was examined in the DT40 B-cell line. In our study, TCDD did not have an effect on the expression of anti-apoptotic factors Bcl-2 and Bcl-xL, between 1 and 6 h post treatment.

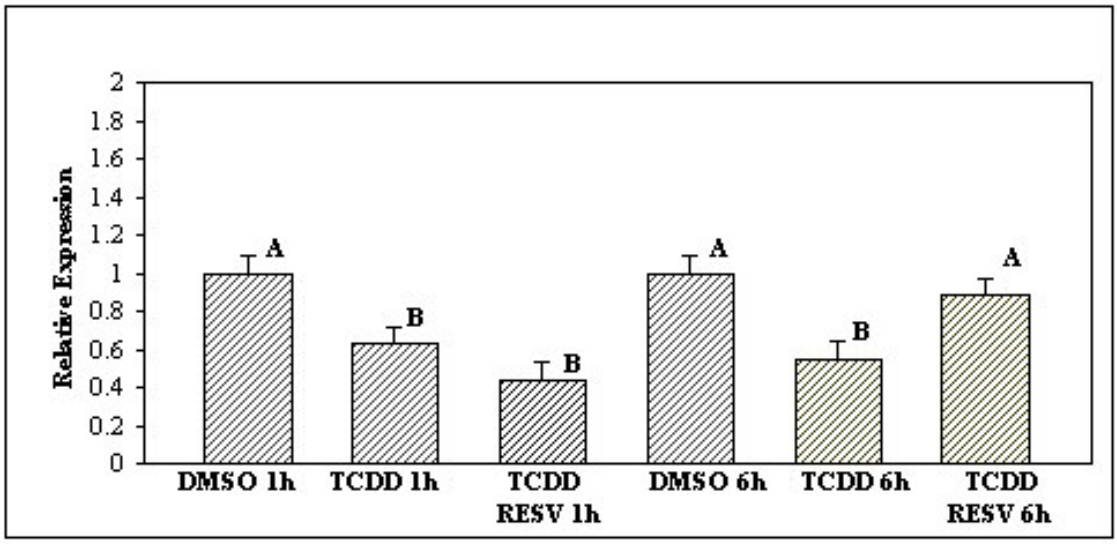

Figure 3.6B. Effect of TCDD (10 nM) on the expression of caspase 3 in DT40 cells after 1 and 6 h exposure with and without pretreatment with resveratrol. Each column represents the average (± standard error) of six ΔΔCT values obtained real time PCR, correspondent to three samples analyzed in duplicate using β-actin as the calibrator. The columns with same letters are not significantly different ($p<0.05$).

Discussion

The immune system is one of the most sensitive of all biologic systems to the adverse effects of TCDD (reviewed by (Kerkvliet, 2002). In mammals, various studies have demonstrated direct effects of TCDD on B-cells, especially on antibody secretion and terminal B-cell differentiation (Inouye et al., 2003; Sulentic et al., 1998; Sulentic et al., 2000), but with only modest effects on B-cell proliferation (Dooley and Holsapple, 1988; Luster et al., 1988). However, a recent study showed TCDD inducible changes in cell morphology and maturation of bone marrow B-lymphocytes (Thurmond and Gasiewicz, 2000). The molecular mechanism of B-cell dysfunction induced by TCDD is not entirely understood because of the myriad of complex mechanisms that involve the

process of differentiation of resting B-cells after antigen activation (Rowlands and Gustafsson, 1997). In avian species, molecular information about TCDD intoxication of B-cells is relatively scarce (Fox and Grasman, 1999; Peden-Adams et al., 1998). Interestingly, both of these studies make reference to considerable loss of viable B-lymphocytes as a consequence of chlorinated hydrocarbon exposure. This led us to hypothesize that changes in the expression of apoptosis-related genes could result from TCDD exposure. Since primary bursal B-cells undergo spontaneous apoptosis within hours after single cell suspension (Neiman et al., 1991), the use of a virally immortalized, well characterized chicken B-cell line provided a reasonable alternative model to test our hypothesis. Despite the presence of the v-rel oncogene in DT40 cells, apoptosis (as assessed by TUNEL assay) was effectively induced after only 6 hours of exposure to TCDD, although a minimal dose of 100 nM was required for this effect to be statistically significant. The increase in number of apoptotic cells in this model (5.5 % vs. 2 % in controls) may seem rather modest, but if extrapolated to a chronic exposure regimen, it could lead to substantial cell loss. In addition, induction of apoptosis is largely cell type-dependent: while TCDD-induced apoptosis has also been reported in human T-lymphoblastic cell line (L-MAT) (Kikuchi et al., 1998) and in Jurkat T-cells (Kwon et al., 2003), similar treatment of human macrophages did not result in apoptotic cell death (van Grevenynghe et al., 2004). The integrity of the signal transduction pathway was verified by induction of cytochrome P450 (CYP1A4), an essential part of the cellular detoxification mechanism in eukaryotic cells. In addition, it appears that TCDD-induced transcription of P450 is repressed by a labile protein, whose synthesis

needs to be blocked to restore hydrocarbon inducibility. This is also the case in a variety of other mesenchymal cell types (Chun et al., 2001; Ou and Ramos, 1995). In combination, these observations validate the use of avian DT40 cells as a model for the study of the gene interactions underlying the effects of TCDD on avian B-cells.

Several genes that participate in the process of signal transduction, starting from the point of ligand-receptor interaction (such as AhR and Arnt) down to executioner molecules (such as caspase 3), were selected for study by high throughput real-time PCR using the ΔΔCT method. This method has been broadly accepted to determine the relative amount of mRNA of a specific target gene under different treatment conditions (Lehmann and Kreipe, 2001; Livak and Schmittgen, 2001; Schmittgen and Zakrajsek, 2000). To strengthen the validity of our findings, the expected amplicon size was verified electrophoretically and six individual samples were included in each treatment group and processed independently.

Upon entry into the cell through the cell membrane, TCDD caused transient upregulation of the mRNA for AhR 1 hour after the initiation of exposure, but not after 6 hours of exposure. A tendency towards upregulation of Arnt mRNA was seen after 1 hour, but became statistically significant only after 6 hours of exposure. Similar upregulation of steady state AhR mRNA by its own ligand has also been observed in an in vivo study in the liver of the rat, not only at the mRNA level but also at the protein level, and at the level of ligand binding (Franc et al., 2001). Our own study did not measure AhR protein levels, but other in vitro studies describe rapid and prolonged depletion of AhR protein by AhR agonists (Giannone et al., 1998; Pollenz, 1996), due to

proteolytic degradation via the ubiquitin-proteasome complex. Downstream genes included in this study were three cysteine-aspartate proteases (caspase 3, 8 and 9).

Caspases, in general, are the principal effectors of apoptosis, and are highly conserved throughout the animal kingdom. Caspases 8 and 9 belong to a group with long N-terminal pro-domains that undergo homotypic interaction with specific adaptor proteins such as FADD/Mort1 or Apaf-1. Caspase 3 belongs to a group of caspases with short pro-domain (caspases 6 and 7 are included in this group) that are activated predominantly through proteolysis by already active caspases (Marsden and Strasser, 2003). In this study, TCDD induced a significant, but transient, increase in gene expression of caspase 9, which is a pivotal player of the caspase cascade. Indeed, caspase 9 directly activates the enzymatic activity of executioner caspases 3 and 6 at the protein level. Although the message of caspase 3 was not consistently upregulated in the present study (it was transiently upregulated by the 100 nM dose, but slightly downregulated by the 10 nM dose), upregulation of caspase 9 mRNA will ultimately result in enhanced caspase 3 executioner activity at the protein level. The fact that this effect was blocked by pre-incubation with resveratrol suggests that the observed caspase inductions were AhR-mediated. Taken together, our data show for the first time that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) causes aryl hydrocarbon receptor (AhR) - mediated apoptosis in the avian B-cells, likely through activation of caspases 9 and 3.

CHAPTER IV

ANALYSIS OF GENE EXPRESSION DURING 2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN (TCDD) - INDUCED TOXICITY IN CHICKEN MACROPHAGES (HD11)

Overview

In this study, we used chicken immune cDNA microarrays (constructed at the Fred Hutchinson Cancer Research Center) to characterize the transcriptional profile induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in chicken macrophages (HD11). The complete array contained 4,186 chicken lymphocyte cDNA spots representing 2,200 genes. Cultures of the chicken myelomonocytic line HD11, a macrophage cell line transformed by the myc-encoding MC29 virus, were exposed to two doses of TCDD (1 and 10 nM) for 6 and 12 h. Cells exposed to a similar amount of DMSO were used as controls. Total RNAs were extracted and the labeled cDNA samples (Cy3 and Cy5) co-hybridized on an individual array. Scanning and image processing were performed with a GenePix 4000 scanner. The resulting images were analyzed using GenePix Pro 4.0. The Log2 values of the median of ratios were used for further analysis. Upon filtering, a total of 173 genes showed significant up- or down regulation, and these were further analyzed using hierarchical clustering (HCL) tree formation, and k-means clustering. In the present investigation, we only present results from HCL and dendrogram. Seven clusters were formed using tree average linkage of genes with similar expression. Downregulated genes after 6 h of exposure to TCDD

(regardless of the dose) and subsequent down-regulation after 12 h of exposure, was observed for the following genes: importin B and importin 7. Consistently down-regulated genes included: inflammatory response-related MMP-2 (matrix metalloproteinase-2), cell cycle-related cullin 2; nitric oxide synthase (NOS); and CRM1/exportin, among others. Specific primers were designed for a selection of these genes and real-time PCR was used for validation of microarray data. The latter experiments are described in Chapter V.

Introduction

The classic dioxin, TCDD, causes a myriad of biological effects that are mediated almost entirely through the activation of the aryl hydrocarbon receptor (AhR) (Schmidt et al., 1996). The pathophysiological role of the receptor has been described in chapter I of this dissertation. Numerous reports have demonstrated the immunosuppressive effects of TCDD at the humoral and cellular levels in several in vivo and in vitro models (Holsapple et al., 1991);(Kerkvliet, 1995; Lang et al., 1998; Oughton et al., 1995).

The immunotoxicity of TCDD is not restricted to B- and T-lymphocytes since studies in the rat (Bagchi and Stohs, 1993) and in the mouse have described the effect of TCDD on peritoneal macrophages. In mice, it was shown that TNF plays a critical role in mediating the TCDD-induced enhanced inflammatory response to intraperitoneal (i.p.) sheep red blood cells (Moos et al., 1997). It has also been demonstrated that AhR was expressed during human monocyte differentiation (Hayashi et al., 1995), and in

human macrophages (Komura et al., 2001). The latter study demonstrated that CYP1A1 mRNA expression is induced in the presence of AhR ligands. TCDD-exposed rats expressed elevated levels of inducible CYP1A1 in the Kupffer cells (in the liver), in (lung) alveolar macrophages and in splenic macrophages, as compared to other macrophage subpopulations or cells from control rats (Germolec et al., 1995).

In this study, we have used commercially available high density cDNA microarrays to initiate the development of a database of genes whose expression is affected by TCDD in macrophages. In order to avoid the genetic variability of primary macrophages as a potential source of variation, and to improve overall reproducibility, this study used an established chicken macrophage line, designated HD-11 (Beug et al., 1979). The readily available chicken immune array developed by the FHCRC as a tool for the study of carcinogenesis in the immune system (Neiman et al., 2001), includes a cDNA library with an average insert size of about 1.5 kb pairs prepared from DT-40 cell line (Abdrakhmanov et al., 2000). It also includes gene-identified clones from chicken activated T-cell cDNA library (Tirunagaru et al., 2000), and 50 selected non-overlapping expressed sequence tag (EST) clones (DKFZ426) from a bursal library developed from a normal 2-week old chicken at the German Resource Center (http://www.rzpd.de). This particular array represented about 2,200 different genes. The use of this array has allowed us to develop a comprehensive database of macrophage genes, affected directly or indirectly by TCDD, that could shed some light on the complex mechanism of toxicity induced by this toxicant. In this study, we have used a variety of tools for

exploring expression data, such as different clustering algorithms (Dysvik and Jonassen, 2001) and visualization methods (Sturn et al., 2002).

Our results reveal several clusters of interacting gene functions that may help provide an explanation for the multiple effects of TCCD and serve as the basis to propose novel experimental hypotheses.

Materials and Methods

Chicken immune cDNA microarray

A chicken immune cDNA microarray was constructed at the FHCRC (Neiman *et al*., 2001). The array included a cDNA library with an average insert size of about 1.5 kb pairs prepared from the DT-40 cell line (Abdrakhmanov et al., 2000); gene-identified clones from a chicken activated T-cell cDNA library (Tirunagaru et al., 2000), and 50 selected non-overlapping expressed sequence tagged (EST) clones (DKFZ426), from a normal 2-week old bursal library from the German Resource Center (http://www.rzpd.de). The complete cDNA microarray contained approximately 4186 transcripts representing about 2,200 different genes.

Cell culture

The HD11 cells (a kind gift of Dr. Bruno Goddeeris, KULeuven, Belgium) are an established chicken myelomonocytic line transformed by the *myc*-encoding MC29 virus, have macrophage-like properties, and express Fc receptors, phagocytic capacity and macrophage cell surface antigens, but only weakly express myeloblast cell surface

antigens. The cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen-Gibco, Carlsbad, CA) supplemented with 8% (v/v) fetal calf serum (Hyclone, Logan, UT) 5% chicken serum (Sigma, St. Louis MO), 100 units/ml penicillin (Sigma), and 100 μg/ml streptomycin (Sigma) at 37 °C and in a 5% CO_2 atmosphere.

Chemical treatment and experimental design

For microarray analysis, cell suspensions were kept for 72 h of incubation; the cells were counted and divided evenly into 16 75-cm^2 culture flasks. An initial number of approximately 1 X 10^5 cells/ml was used. The cells were incubated for an additional 48 h and then six flasks were exposed separately (three flasks per time point) to 1 and 10 nM of TCDD (Sigma) solubilized in dimethyl sulphoxide (DMSO; Sigma), for 6 and 12 h. The remaining control flasks were exposed to the same volume of DMSO. Cells were harvested from the TCDD-exposed and the control flasks at 6 and 12 h post-exposure, respectively. Cells from each flask were processed individually for total RNA extraction. The total RNA from individual flasks was then pooled within treatments for microarray hybridization.

RNA extraction

The cells from each sample were pelleted and processed for total RNA extraction using the Trizol® (Invitrogen, Carlsbad, CA) method. Total RNA concentrations were determined by OD_{260}.

Sample preparation and hybridization

Labeling of cDNA samples, preparation of microarrays, and hybridization reactions were performed, as a custom service, by the FHCRC. Here, for informative purposes, the techniques are briefly described. The total extracted RNA was linearly amplified using a T7 promoter-based method (Ambion, Inc., Austin, TX) and use of a random prime labeling methodology. A minimum of 30 μg of each pool of total mRNA was used, and then transformed into cDNA. Target cDNAs from control RNA samples were labeled by reverse transcription using Cyanine-3 (Cy3) and the TCDD-exposed samples were labeled with Cyanine-5 (Cy5). The coupling of both fluorophores was carried out using an established amino-allyl coupling procedure. The Cy3- and Cy5-labeled cDNA samples were combined and co-hybridized on the same array for 16 hrs at 63°C, followed by a sequential, stringent washing procedure (Fazzio et al., 2001).

Image scanning and data processing

Hybridized slides were scanned using a GenePix 4000 Microarray scanner (Axon Instruments, Inc., Union City, CA). Two 16-bit tiff images were collected for each array corresponding to 532 nm (Cy3) and 635 nm (Cy5) excitation wavelengths. Image analysis was performed using GenePix Pro 4.0 software (Axon Instruments, Inc., Union City, CA). Normalization of Cy5 to Cy3 signal per experiment was determined assuming equivalent global hybridization of test and reference probes to genomic chicken DNA.

Values of Log2 of the ratio of medians were filtered using the max/min criterium, which corresponded to two standard deviations above or below zero. The resulting values were processed using GENESIS (Institute for of Biomedical Engineering, Graz University of Technology).

Determining repression or induction

Fluorescent ratios on a microarray are optically analyzed to determine whether there has been repression or induction of any particular gene. The quantification of both fluorophores (red and green) is carried out by image scanning. In the present experiment green represented the control mRNA and red represented the experimental mRNA. A calculated ratio of red:green tells whether a particular gene was induced or repressed as a consequence of the experimental treatment in comparison to the baseline expression (of the non-treated cells) . Then, the expression ratio is calculated as a decimal value (*e.g.* 0.2 for a 5-fold reduction), and the decimal value is then transformed into logarithmic (base 2) scale, which makes it easier to visualize induction versus repression (Gasch et al., 2000).

$$\textit{Ratio: red : green} \quad \rightarrow \quad \textit{red} \div \textit{green} \quad \rightarrow \quad \log_2 (\textit{red} \div \textit{green})$$

If the expression of the experimental sample is greater than the control, the ratio will be greater than one, and then the final number after logarithmic transformation will be positive. The final number will be negative if the expression of the experimental sample is less than the control, and so if the ratio of the experimental sample and the control is 1:1, then the result will be zero. Thus, to easily assess the repression or induction, the

resulting data are converted into images using red and green as artificial colors. Because the same colors (red and green) have been used in the original hybridization experiment, this could be a source of confusion. It is very important not to forget that the artificial colors are just a mere representation of changes in expression and do not represent the initial fluorescence from the mRNA hybridization experiment. In a typical scale, a black spot on the array would indicate that equal amounts of red and green fluorescence were observed on the original spot, thereby giving an equal expression ratio of 1:1, and subsequently a logarithmic value of 0.

Clustering

The process of separating a set of objects, according to their similarity, into several subsets, is called clustering (Gilbert et al., 2000). In general, two strategies are possible: supervised (based on existing knowledge) and unsupervised clustering. In supervised clustering, existing biological information is used to guide the classification of genes affected by chemical treatment. Unsupervised clustering is used to define clusters that minimize intra-cluster variability while maximizing inter-cluster distances, i.e. finding clusters, whose members are similar to each other, but distant to members of other clusters in terms of gene expression based on the used similarity measurement.

Hierarchical clustering is referred to the method that identifies a small group of genes that share a common pattern of expression and then constructs the dendrogram in a sequential manner using a ranked series of clusters. Non-hierarchical clustering, such as k-means analysis, assigns each gene expression datum to a cluster based on its

expression profile, and then repeats the process until every datum point has been placed in a cluster (Schena, 2003).

Results

Analysis of expression during TCDD exposure

Chicken macrophages were subcultured for analysis of gene expression upon exposure to TCDD (1 and 10 nM) for 6 and 12 h. This manipulation resulted in a significant change of gene expression of at least 173 genes out of an approximate 4,000 clones. As mentioned above, the cut-off to select those genes was set at two standard deviations from the mean. The genes were clustered according to the their expression value using hierarchical clustering algorithms (Gilbert et al., 2000). Consequently, seven clusters were created (Fig. 4.1, 4.2); especially, clusters B through G displayed a characteristic profile as a function of time, but not as a function of dose. With both TCDD doses (1 and 10 nM), significant transient upregulation is observed after 6 hours of exposure. In contrast, 6 hours later (i.e. after 12 hours of TCDD exposure) the very same genes were significantly downregulated. Fig. 4.3 shows the annotation of the first part of the dendrogram shown in Fig. 4.1 (clusters A through D). It visualizes relative expression levels (in artificial color); lists the GeneBank accession numbers and a brief description of the gene function, as far as it is known.

Table 4.1 lists a selection of genes that were further analyzed by data mining, which includes comparing the sequence of the microarray clones with the public database (using BLAST on NCBI), in order to obtain more up to date information about

the function of the gene involved. Some genes (Importin B and importin 7) display the typical profile described above (up-regulation after 6 hours, down-regulation after 12 hours of TCDD exposure, regardless of dose). However, the microarray analysis also revealed a number of genes that were constantly and fairly dramatically down-regulated; a "-3" in this table signifies a 2^3-, i.e. 8-fold down-regulation. These genes include cullin 2 (cell cycle-related; (Kipreos et al., 1996), nitric oxide synthase (NOS) (which has a variety of vascular and immune functions; (Lowenstein et al., 1992); CRM1/Exportin 1 (involved in nuclear transport and chromosome maintenance; (Stade et al., 1997) and, most importantly, matrix metalloprotease-2 (extracellular matrix degradation and inflammation; (Goetzl et al., 1996). The latter finding provided the basis of the follow-up investigation described in chapter V that deals with the detrimental effect of TCDD on the expression of matrix metalloproteases MMP-2 and MMP-9 in cells pre-exposed to TCDD, especially when challenged with a pro-inflammatory molecule (LPS).

Table 4.1. Selected genes from analysis of microarray of HD11 chicken macrophages exposed to TCDD. Induced changes in expression involve apoptosis, nuclear transport, metabolism, cell cycle regulation, and inflammation, among others.

Gene Bank Number	Gene name	Induced				Repressed				Function	Reference
		A	B	C	D	A	B	C	D		
602738	Importin beta	1.2		0.6			-1.7		-1.2	Nuclear transport	(Kutay et al., 1997)
123970	Cytochrome C	1.6		1.4	1.3		-2.5			Electron transport, apoptosis	(Liu et al., 1996)
730163	RAN 7/importin 7	2.8		1.6				-4.5	-2.7	Nuclear transport	(Gorlich et al., 1997)
603135	Cullin 2					-2.9	-1.3	-1.5	-1.5	Cell cycle	(Kipreos et al., 1996)
163731	Nitric Oxide Synthase					-2.4	-1.8	-2.7	-1.5	Vascular and immune function	(Lowenstein et al., 1992)
602559	CRM1/Exportin 1; XPO1					-0.9	-2.9	-1.4	-2.5	Chromosome maintenance	(Stade et al., 1997)
120360	Matrix metalloprotease -2					-3.1	-3.7	-3.1	-3.0	ECM degradation, inflammation	(Goetzl et al., 1996)

Notes for treatments: A) 1 nM TCDD for 6 h; B) 1 nM TCDD for 12 h; C) 10 nM TCDD for 6 h; D) 10 nM TCDD for 12 h.

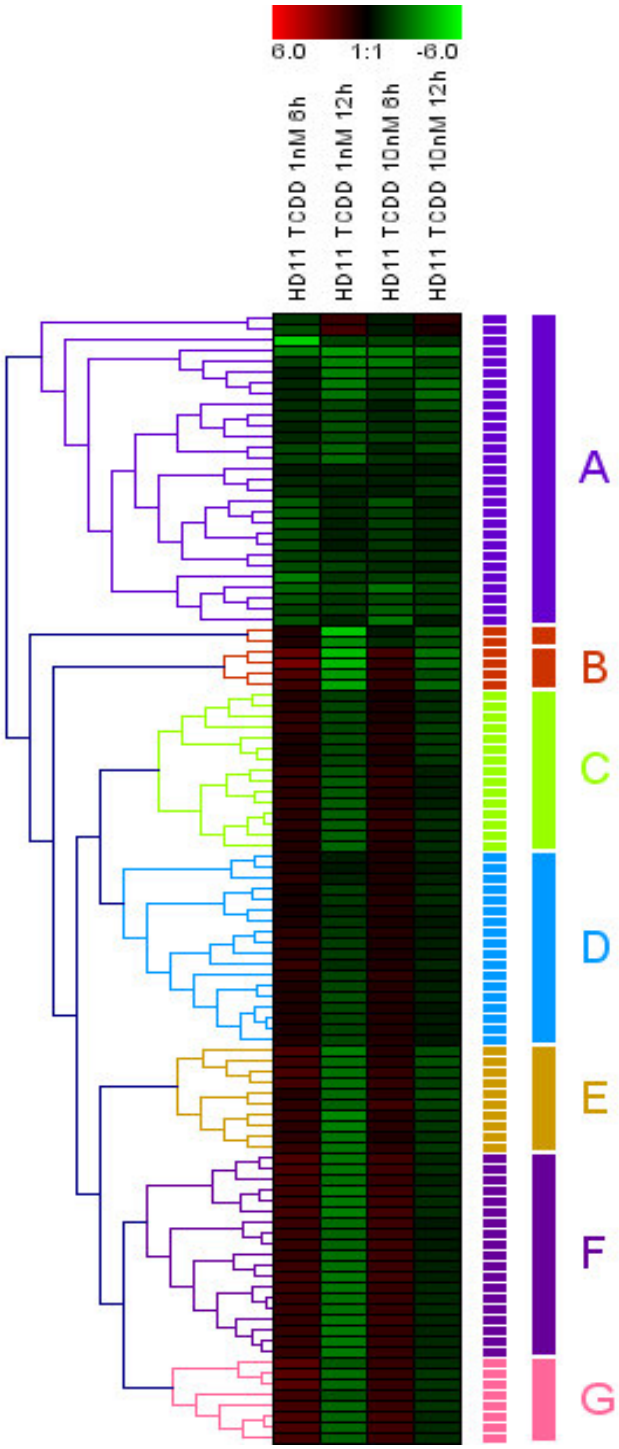

Figure 4.1. Cluster analysis of microarray data from HD11 chicken macrophages exposed to 1 nM TCDD during 6 and 12 h (left columns) and 10 nM during the same period of time (right columns). The tree (dendrogram) is the result of hierarchical clustering analysis using average linkage between genes. The analysis was performed using GENESIS microarray software (http://genome.tugraz.at/Software/GenesisCenter.html).

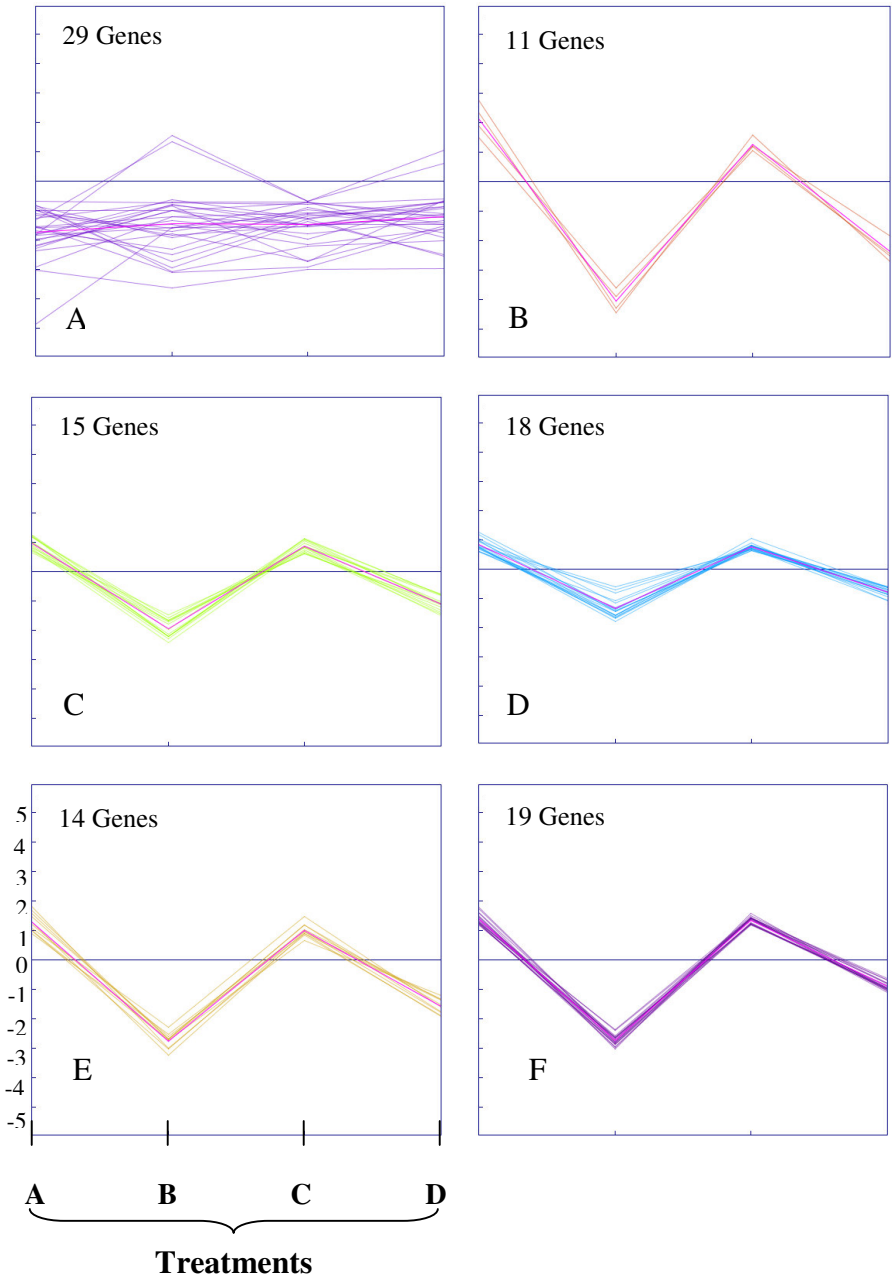

Figure 4.2. Gene expression profile using hierarchical clustering (HCL) in HD11 cells exposed to TCDD. The HCL method identifies a small group of genes that share a common pattern of expression, then constructs the dendrogram in a sequential manner using a ranked series of clusters (Schena, 2003). The positive numbers indicate the up-regulated genes, and the negative indicate the down-regulated genes. The treatments were as follows: A: 1 nM for 6h; B: 1 nM for 12 h; C: 10 nM for 6 h; and D: 1 nM for 12 h.

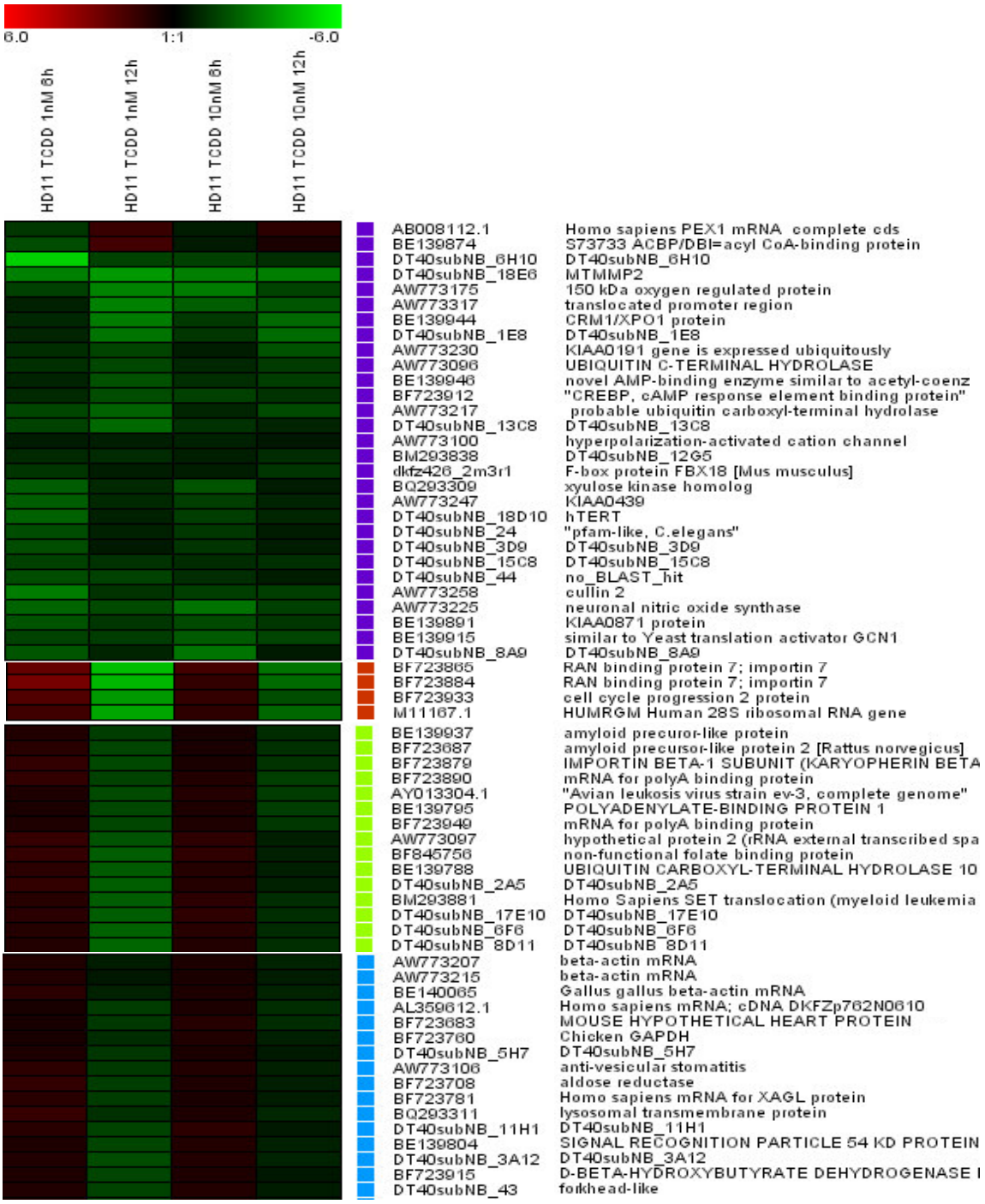

Figure 4.3. Clustering analysis of the gene expression profile of HD11 macrophages exposed tc TCDD. The genes were classified according to their average expression. This panel represents from top cluster A, B, C, and D. Colored columns shows the treatment groups, the single bar identifies each cluster, the third column shows the GeneBank accession number, and the fourth column shows the description for each gene, as far as it is known.

Discussion

TCDD has been demonstrated to suppress the humoral and cellular immune response, and to possibly increase the susceptibility of animals to several infectious diseases and other dysfunctions (Kerkvliet, 2002). The biological effects of TCDD have been correlated with its ability to bind and activate the aryl hydrocarbon receptor (AhR).

Following AhR activation, the transcriptional response of an impressive number of genes is altered, thus producing extensive toxic effects (Puga et al., 2000). It has been demonstrated that macrophages are functionally compromised by direct exposure to TCDD, and that AhR is expressed during human monocyte differentiation (Hayashi et al., 1995). In human macrophages for instance, AhR is expressed and CYP1A1 mRNA expression is induced in the presence of AhR ligands (Komura et al., 2001). Studies in rat (Bagchi and Stohs, 1993) and mice have described the effect of TCDD on peritoneal macrophages. In mice it was shown that TNF plays a critical role in mediating the TCDD-induced enhanced inflammatory response to intraperitoneal (i.p.) sheep red blood cells (1997). TCDD-treated rats expressed elevated levels of inducible CYP1A1 in Kupffer cells (in the liver), (lung) alveolar macrophages and splenic macrophages as compared to other macrophage subpopulations or cells from control rats (Germolec et al., 1995).

In this study, we have analyzed the transcriptional changes in the chicken macrophage HD11 cell line induced by exposure to 1 and 10 nM TCDD for 6 and 12 hours. HD11 cells have been transformed by MC29 virus and have macrophage-like properties; they strongly express Fc receptors, phagocytic capacity and macrophage cell

surface antigens, but only weakly express myeloblast cell surface antigens (Beug et al., 1979). They have been not previously been used for immunotoxicological studies involving AhR ligands.

In this investigation, we have observed important shifts in gene expression as a result of exposure to TCDD. Even though we still encountered the same limitations as described in chapter II of this dissertation (especially lack of annotation), this in vitro macrophage model showed more consistent and more exciting results than the DT40 B-cell model. From the clones that were selected by GENESIS as clones of interest (based on the fact that their expression level deviated more than two standard deviations from the average expression level of all spots on the array), a number of genes were essential to macrophage function. Hence, the results from the present investigation were used as a corner stone for the next chapter, which deals with the effect of TCDD on the expression of matrix metalloproteases MMP-2 and MMP-9, also known as gelatinase A and B, respectively (Nagase et al., 1992). These two proteases consistently showed an approximate 8-fold downregulation below control levels in all experimental conditions tested (see Table 4.1). Surprisingly, the effects of TCDD on the gene expression of MMP-2 and MMP-9 have not been described so far.

The few reports mentioning the effect of TCDD on any member of the metalloproteases family include the effect on MMP-1 in keratinocytes (Murphy et al., 2004), and the disruptive effect of TCDD on steroid regulation of endometrial matrix metalloproteinase (MMP) expression (Bruner-Tran et al., 1999).

Several other genes were also consistently downregulated in all experimental conditions. Cullin 2 is involved in cell cycle regulation; null mutations of the gene were reported to cause hyperplasia of all tissues in nematodes. G1-to-S phase progression was accelerated, overriding mechanisms for mitotic arrest and producing abnormally small cells (Kipreos et al., 1996). This may be the molecular basis of the hyperplasia of keratinocytes (Greenlee et al., 1985) as observed in chloracne. Nitric oxide synthase (NOS), is a gene related to cardiovascular, neuronal and cellular immune function (Lowenstein et al., 1992). In macrophages, NOS represents an essential killing mechanism used in the defense against pathogens (Dil and Qureshi, 2002a). Consistent downregulation of this enzyme might therefore have serious consequences for the effectiveness of innate immune function. Finally, CRM1/exportin is involved in nuclear transport, chromosome maintenance, and cell proliferation (Stade et al., 1997).

Evidence in HeLa cells suggests that CRM-1 and XAP-2 act in parallel through different mechanisms and target different interfaces of the receptor. Those results imply that two pathways cooperate to localize the non-activated receptor in the cytoplasmic compartment of the cell (Berg and Pongratz, 2002).

In conclusion, although several interesting working hypotheses could be (and were) distilled from the presented microarray data, time constraints made it impossible to corroborate and refine each of those hypotheses by use of alternative techniques such as real-time PCR. Therefore, we decided to pursue the working hypothesis that exposure to TCDD might induce decrease in gene expression of MMP-2 and MMP-9 in the chicken macrophage cell line HD11 (see Chapter V). Despite the limitations of the microarray pointed out previously (chapter II), there is little doubt that further data mining of the database of clones that responded to TCDD exposure has the potential to generate additional working hypotheses and data mining should thus be further pursued.

CHAPTER V

2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN (TCDD) IMPAIRS THE MMP-2 AND MMP-9 RESPONSE TO LPS STIMULATION IN HD11 CHICKEN MACROPHAGES

Overview

Xenobiotic AhR ligands are notorious for their immunosuppressive actions, targeting not only B- and T-lymphocytes but also antigen-presenting cells, such as macrophages. This study describes a new aspect of the negative impact of TCDD on macrophage function, namely, an impairment of their capacity to respond to an inflammatory stimulus with secretion of a panel of proteolytic enzymes, collectively termed matrix metalloproteases. Our previous microarray experiments using the HD11 chicken macrophage line have suggested a consistent downregulation of the inflammatory response-related matrix metalloprotease gene MMP-2 after exposure to 1 and 10 nM of TCDD for 6 and 12 h. To corroborate these preliminary findings, cultures of the chicken myelomonocytic cell line HD11 were exposed to 10 nM of TCDD for 24 h, and the gene expression profiles of MMP-2, MMP-9, AhR and CYP1A4 were investigated with or without stimulation with LPS (10 μg/ml) for 6 h following exposure to TCDD. Upregulation of CYP1A4 by more than one order of magnitude, clearly demonstrated the integrity of the downstream signaling pathway initiated by binding of TCDD to the cytosolic AhR. A first important conclusion from these studies was that LPS-stimulated macrophages appeared to be much more sensitive to TCDD exposure

than resting macrophages, judged from the 7-fold upregulation of the AhR message in response to LPS activation. Moreover, a synergistic effect between TCDD exposure and LPS stimulation on the upregulation of the AhR message was observed. While a 24 – hour exposure to 10 nM TCDD did not reduce MMP levels as judged by real-time RT-PCR, TCDD dramatically decreased the normal upregulation of both MMP-2 and MMP-9 in response to LPS stimulation. LPS-induced upregulation of MMP-2 and MMP-9 expression in macrophages not previously exposed to TCDD was 12- and 20-fold, respectively, while pre-exposure to TCDD caused this response to drop to 3-fold and 7-fold, respectively. We could not observed the effect of TCDD on the expression of MMP-2 and MMP-9 at the protein level after 6 h stimulation with LPS. Resveratrol blockage of the AhR prior to TCDD exposure and LPS stimulation of the HD11 cells showed that the AhR is involved in the upregulation of its own message. Similar conclusions with regard to MMP regulation could not be drawn, since resveratrol by itself has been reported to significantly downregulate MMP expression in other cell types. TCDD-induced downregulation of MMP-2 and MMP-9 demonstrate for the first time that TCDD likely disrupts the *de novo* synthesis of MMP-2 and MMP-9 in macrophages upon stimulation with a pro-inflammatory mediator (LPS). As a consequence, exposure to dioxin may impair important processes in tissue remodeling during tumor necrosis, and inflammation.

Introduction

2,3,7,8-tetra-chlorodibenzo-p-dioxin (TCDD) is considered a potent contaminant that belongs to a large group of halogenated aromatic hydrocarbons, ubiquitous and persistent environmental contaminants. Most of the biological effects of this group of compounds are mediated by the activation of the aryl hydrocarbon receptor (AhR)(Schmidt and Bradfield, 1996). The AhR is a member of the basic helix-loop-helix (bHLH) and PAS (Per/Arnt/Sim) domain family of proteins (Whitlock, 1999).

Upon binding to its ligand in the cytosol, the AhR dissociates from two 90 kD heat shock proteins and the Hepatitis virus X-associated protein 2 (XAP2). The receptor-ligand complex then translocates into the nucleus, where the AhR dissociates and dimerizes with the aryl hydrocarbon receptor nuclear translocator (Arnt), forming a heterodimer (Carver and Bradfield, 1997). The heterodimer interacts with dioxin responsive elements (DRE) located in the promoter region of target genes inducing their activation or repression. These DRE-containing genes include metabolic enzymes (Nebert, 1994), growth factors (Kim et al., 2000; Zaher et al., 1998) and cytokines (Lai et al., 1996). A myriad of disorders are associated with AhR, including immunosuppression, thymic atrophy, cleft palate, atherosclerosis, chloracne, reproductive dysfunction and certain types of cancer (Kim et al., 2000; Puga et al., 2000; Zaher et al., 1998). In birds, the AhR basic helix-loop-helix and the PAS domains exhibit more than 90 % and 85 % sequence similarity respectively, with that of the mammalian Ah receptor (Walker et al., 2000). In addition, these authors showed that the chicken AhR dimerizes with human AhR nuclear translocator and binds to the

mammalian dioxin-response element (DRE) in a ligand-dependent manner. The cytochrome P450 isoform 1A4 (CYP1A4), the avian homologue of CYP1A1 (Gilday et al., 1998) was highly induced by TCDD in a subset of tissues expressing AhR. The highly conserved nature of DREs and AhRs at the level of DNA binding was also reported by earlier studies on the species-specific binding of the transformed AhR to the DRE (Bank et al., 1992). Transcriptional profiles in HepG2 cells exposed to TCDD, have shown altered expression patterns for more than 300 known genes. Those genes were classified according to their physiological relevance including genes involved in regulation of the cell cycle, differentiation, apoptosis, development, cell adhesion, cancer and metastasis (Puga et al., 2000).

As mentioned above, many xenobiotic AhR ligands are potent immunosuppressive toxicants. Although both B- and T-lymphocytes have been shown to be targets (reviewed by Holsapple (Holsapple et al., 1991; Hossain et al., 1998; Puebla-Osorio et al., 2004) evidence is accumulating that monocyte-derived cells are also implicated in the immunotoxicity of dioxin. Various functions of macrophages, such as antigen presentation, interaction with T-cells through cytokine production and phagocytic activity are impaired by polycyclic aromatic hydrocarbons (Myers et al., 1988; Myers et al., 1987; Tewari et al., 1979). This paper deals with a macrophage function that, to the best of our knowledge, never has been implicated in the immunosuppressive functions of PAH, namely the capacity to degrade connective tissues for the purpose of remodeling by secretion of matrix metalloproteases (MMPs).

The matrix metalloproteases 72- and 92-kD type IV collagenases are members of a group of secreted zinc metalloproteases that degrade collagens within the extracellular matrix. The 72-kD type IV collagenase (MMP-2 or CLG4A) is secreted from macrophages (Goetzl et al., 1996) and connective tissue (Ito et al., 1996). Ito et al. (1996) have shown that matrix metalloproteinases (MMPs) and interleukin 1 (IL-1), a potent stimulator of connective tissue cells to produce MMPs, are implicated in inflammation and tissue destruction. These authors demonstrated that activated MMP-2 and MMP-9 negatively regulate IL-1β, but not IL-1α, suggesting this negative feedback loop as a regulatory mechanism of MMPs. The 92-kD collagenase (MMP-9 or CLG4B) is produced by normal alveolar macrophages and granulocytes (Nagase et al., 1992). (Van den Steen et al., 2000). Van den Steen et al. (2000) have demonstrated that MMP-9-mediated N-terminal cleavage of IL-8 potentiates IL-8 activation of neutrophils, as measured by increased intracellular calcium concentrations, MMP-9 secretion, and neutrophil chemotaxis.

In the present study, we have analyzed the mRNA expression of the chicken matrix metalloproteases MMP-2 and MMP-9 in HD11 chicken macrophages exposed for 24 h to 10 nM of TCDD, TCDD followed by LPS stimulation, or DMSO as negative control. The ligand-related genes AhR and CYP1A4 were also analyzed.

Materials and Methods

Cell culture

The model used in this study consisted of the chicken myelomonocytic cell line (HD11) transformed by the *myc*-encoding MC29 virus (Beug et al., 1979). Cell cultures were propagated in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM L-glutamine; 10% (v/v) fetal bovine serum; and 5% (v/v) chicken serum, and 100 U of penicillin and 10 mg of streptomycin per ml, cultured at 37 ºC in a 5% CO_2-balanced air environment in 75 cm^2 culture flasks.

Chemical treatments

TCDD concentrations and exposure times were based on the literature (Matikainen et al., 2001; Tian et al., 1999). HD11 cell cultures were counted and plated in 6-well plates at a density of approximately 1 x 10^6 cells per well. Plates were incubated for an additional 24 h and then exposed to 10 nM of TCDD, solubilized in dimethyl sulphoxide (DMSO), during 6, 12 and 24 h, or an equivalent volume of DMSO. After TCDD exposure, the cells were activated with 10 µg/ml of lipopolysaccharide (LPS) from *Salmonella enteriditis* (Sigma, St. Louis, MO, product number L-6011) for 6 hours. At the end of the respective treatments, cells were harvested and wells were processed individually for total RNA extraction.

MMP activity assay (Zymogram)

MMP activity was measured in HD11 macrophage-conditioned media after each treatment. The supernatants were electrophoresed under non-reducing conditions (Laemmli, 1970) into 10% Zymogram gelatin gels (BIO-RAD, Hercules, CA). After electrophoresis, gels were washed twice with 2.5% (v/v) Triton X-100 (37°C, 20 min) and incubated for 16 h with development buffer (40 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, and 0.005% (v/v) Brij 35). Gels were stained with 0.5% (w/v) Coomassie blue in 50% methanol and 10% acetic acid for 1 h, and then destained with the methanol/acetic acid mixture. MMPs were detected as clear bands against a blue background. The gel was scanned using the Fluor-S MultiImager scanner (BIO-RAD, Hercules, CA). The density of three bands per experiment was calculated using Quantity One 4.0 software (BIO-RAD, Hercules, CA).

RNA extraction and cDNA synthesis

Total RNA was extracted from each group of cells using Trizol® (Invitrogen, Carlsbad, CA). Total RNA concentrations were determined by OD_{260}. Poly-A^+ RNA was purified using Oligotex® (Qiagen, Valencia, CA). cDNA synthesis was carried out using the 2-Step reverse transcriptase polymerase chain reaction (RT-PCR) method (RETROscript Protocol; Ambion, Austin, TX). Briefly, 100 ng of Poly-A^+ mRNA was combined with 1 µl of oligo dT (50 µM) and denatured at 70 ºC for 10 min. Then, 1 µl of RNAse inhibitor (10 U/µl), 2 µl 10X RT buffer, 4 µl of dNTP mix (2.5 µM each dNTP final concentration), and 1 µl of MMLV reverse transcriptase (100 U/µl) were

added to a final volume of 20 µl. The mix was incubated at 42 ºC for 60 min. The resulting cDNA was denatured at 92 ºC for 10 min and stored at –80 ºC.

Real-time polymerase chain reaction (RT-PCR)

Real-time PCR amplification was performed using the ABI Prism 7700 Sequence Detection System (PE-Applied Biosystems), in 96- well plates. Primers used for the PCR (Table 5.1) were designed using the GeneFisher on-line resource (http://bibiserv.techfak.uni-bielefeld.de/genefisher/). Each primer sequence was verified for uniqueness by comparison with genomic sequences using BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The target genes included is this study were: chicken MMP-2, MMP-9, AhR, CYP1A4, and β-actin as endogenous control.

Each reaction included 0.5 µg of cDNA sample, 12 µl of SYBR Green master mix (PE-Applied Biosystems) and 1 µM of the appropriate gene-specific forward and reverse primers, in a final reaction volume of 20 µl. The thermal cycling conditions included an initial denaturing step at 95^{o}C for 10 min, 50 cycles at 95^{o}C for 15 s, and 65^{o}C for 1 min. All experiments were performed in triplicate for each data point. Each of the averages shown in the figures is the average of three samples, each run in duplicate totaling six samples per group.

To verify the amplicon size for all target genes, the products from the real-time PCR were run on a NuSieve 3:1 (Cambrex Bio Science, Rockland, ME) agarose gel and visualized with ethidium bromide. The effect on the relative gene expression that

resulted from TCDD-exposure of HD11 cells was calculated using the ΔΔCT method (Lehmann and Kreipe, 2001; Livak and Schmittgen, 2001).

Statistical analysis

The average $2^{-\Delta\Delta CT}$ values per treatment included six observations per group at each time point. Analysis of variance was performed, and statistical differences between means were calculated using least-squares means (LSM) ($p<0.05$) (SAS Institute, Cary, NC, USA).

Results

Validation of the HD11 macrophage model

For relative quantification of target genes, triplicate cDNAs from each experimental condition were amplified using primers for chicken MMP-2, MMP-9, AhR, CYP1A4, and β-actin (see Table 5.1). Amplification of each single product was verified for each sample by running a heat dissociation protocol after the final RT-PCR cycle (Morrison et al., 1998; Ririe et al., 1997).

Table 5.1. Real-time PCR primers.

Gene name	Accession Number	Forward	Reverse	Size
cMMP-2	NM_204420	5'CTACCCTTGGACCTGTCA3'	5'GCCAGAATGTAGCAACGA3'	147
cMMP-9	AF222690	5'GTACAGCTACGTCCAGGA3'	5'CCTCCTCTGTGGTGGAA3'	150
cAhR	AF192502	5'ACACCTACTGGCTGTGA3'	5'GACAGTCATTCCACTCTCA3'	146
cCYP1A4	X99453	5'CCTGTCCTGGTGCATGATGTAC3'	5'TCTGATCCAGCTCTGCCTGAA3'	74
cβ-actin	L08165	5'CTGATGGTCAGGTCATCACCATT3'	5'TACCCAAGAAAGATGGCTGGAA3'	100

The primers were designed from chicken gene sequences. Gene access numbers can be verified at http://www.ncbi.nlm.nih.gov/. Each primer sequence was verified for uniqueness by comparison with genomic sequences using BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

The analyses of the CT values obtained from real time PCR assays were performed using the ΔΔCT method. This method has been broadly accepted to determine the relative amount of mRNA of a specific target gene under different treatment conditions (Lehmann and Kreipe, 2001; Livak and Schmittgen, 2001; Schmittgen et al., 2000). To support the validity of the findings, the expected amplicon size was verified electrophoretically; and for the statistical validation, four individual samples were included in each treatment group and processed in duplicate.

The melting curve for each particular target consistently produced a single dissociation peak for each of the target genes and for the reference gene (data not shown). Additional confirmation of the specificity of the amplification reactions was obtained by NuSieve gel electrophoresis (Fig. 5.1).

Exposure of resting HD11 chicken macrophages to TCDD alone caused a dramatic (approx. 13-fold) increase of the relative expression of cytochrome P450 isoform 1A4 (CYP1A4) (Fig. 5.2). TCDD exposure followed by stimulation with LPS further increased the relative expression of CYP1A4, up to 17-fold above control levels (i.e., DMSO alone).

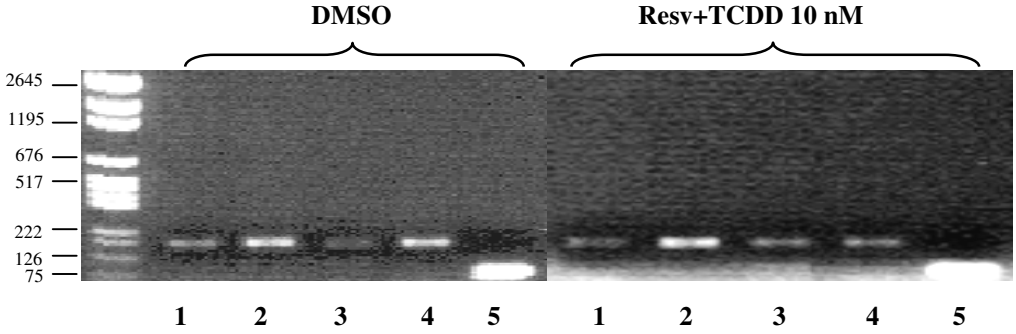

Figure 5.1. Amplicon size of all target genes was verified on a 2% agarose gel. Lanes: 1, AhR; 2, MMP-9; 3, MMP-2; 4, CYP1A4; 5, β-Actin.

In contrast, stimulation with LPS without prior exposure to TCDD did not cause induction of CYP1A4 (Fig. 5.2). The induction of this gene proved that HD11 cells, are highly sensitive to TCDD exposure and a synergistic effect is observed when cells are stimulated with LPS after exposure to TCDD.

AhR expression

As shown in Fig. 5.3, exposure to 10 nM TCDD for 24 hours did not influence the relative expression of the AhR in resting macrophages. Stimulation with LPS

increased the expression of the AhR more than 7-fold above control levels, suggesting that stimulated macrophages may be more sensitive to TCDD than resting macrophages.

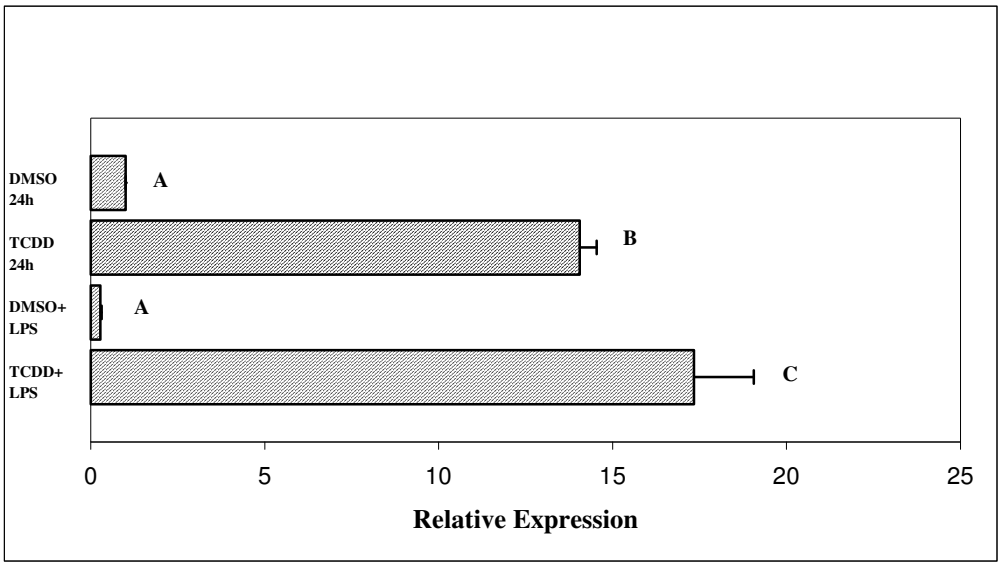

Figure 5.2. Effect of TCDD (10 nM) and LPS on the expression of CYP1A4 in chicken macrophages (HD11). Real-Time PCR results after analysis using the ΔΔCT method. TCDD+LPS treated cells showed a dramatic increase of CYP1A4 induction as compared to TCDD and DMSO alone. The bars correspond to the mean of four samples (run in duplicate) ± standard error. The columns with same letters are not significantly different ($p<0.05$).

Exposure to 10 nM TCDD followed by activation with LPS (10 μg/ml) further increased the relative expression of AhR mRNA. An 18-fold increase of AhR expression was observed after 24 exposure to TCDD followed by 6 h exposure to LPS [18.6 ± 1.77 (SD) vs. 1.0; $P<0.05$] (Fig. 5.3). Pretreatment of the cells with resveratrol (3,5,4′-trihydroxy-*trans*-stilbene), a competitive antagonist of the AhR (Amakura et al., 2003),

was used to assess the involvement of the AhR in TCDD-induced gene expression alterations.

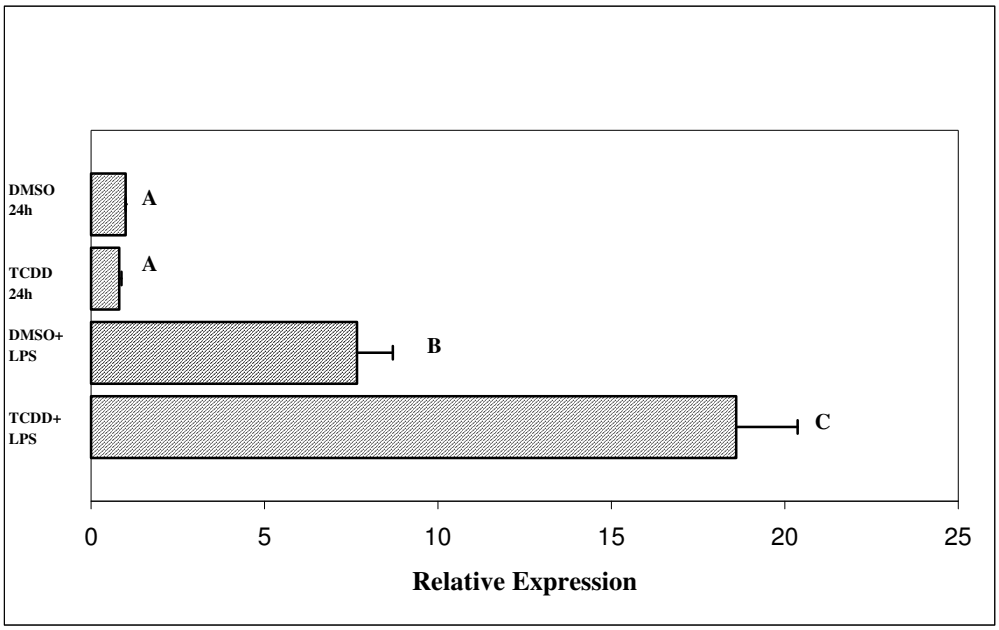

Figure 5.3. Effect of TCDD (10 nM) and LPS on the expression of AhR in chicken macrophages (HD11). Real-Time PCR results after analysis using the ΔΔCT method. TCDD+LPS treated cells showed at least two-fold induction of AhR gene on the TCDD+LPS-exposed cells as compared to TCDD and DMSO alone. The bars correspond to the mean of four samples (run in duplicate) ± standard error. The columns with same letters are not significantly different ($p<0.05$).

A two-fold reduction in the relative expression of AhR was observed in the group pre-incubated for 1 h with resveratrol prior to TCDD exposure; this decrease was similar to that observed in the group pretreated with resveratrol and then exposed to TCDD prior to stimulation with LPS (Fig. 5.4).

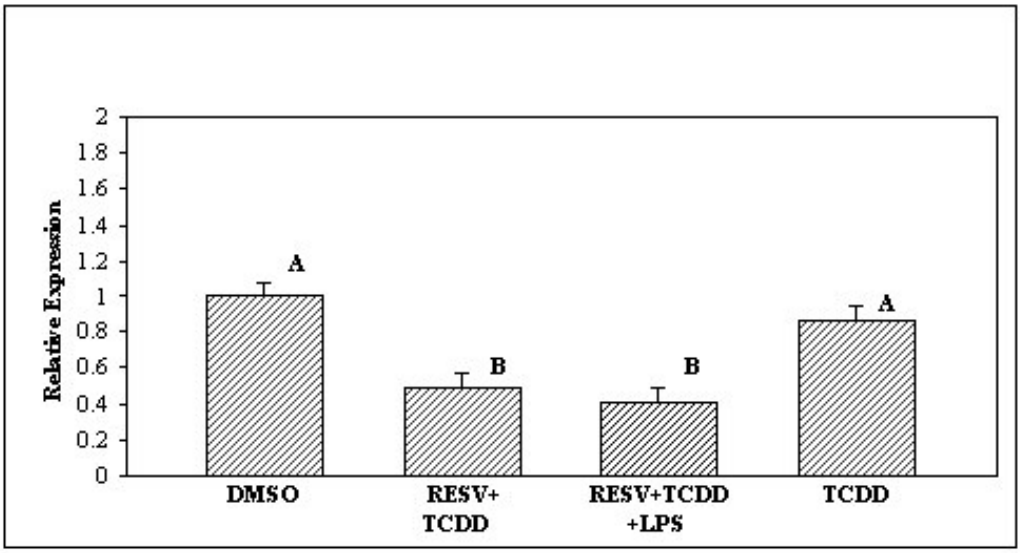

Figure 5.4. Effect of TCDD (10 nM) alone or in the presence of resveratrol on the expression of AhR in HD11 cells. Relative expression of AhR following 6 h of LPS stimulation of cells treated with 10 nM TCDD for 24 h. Resveratrol at 5 μM was administered 1 h prior exposure to TCDD. Each column represents the average (±SE) of ΔΔCT values obtained by real-time PCR correspondent to four samples in duplicate using β-actin as the calibrator.

MMP-2 and MMP-9 gene expression

Macrophages are the major immune source of MMPs, especially when activated by pro-inflammatory mediators, such as LPS (Goetzl et al., 1996). While exposure to TCDD did not significantly influence baseline expression of MMP-2, TCDD clearly inhibited the normal increase in MMP-2 caused by stimulation with LPS (Fig. 5.5).

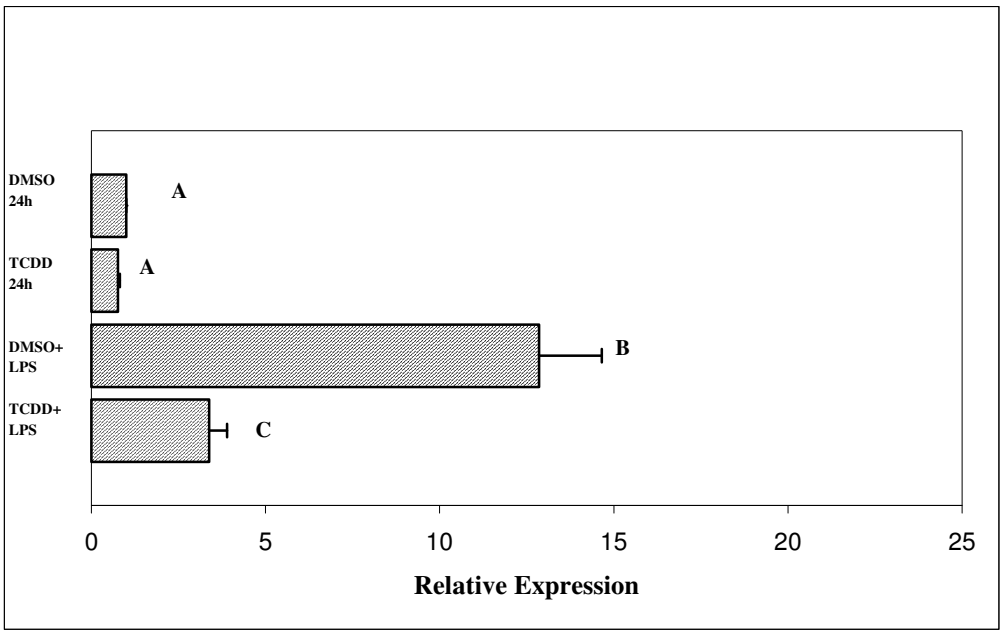

Figure 5.5. Effect of TCDD (10 nM) and LPS on the expression of MMP-2 in chicken macrophages (HD11). Real-Time PCR results after analysis using the ΔΔCT method. TCDD+LPS treated cells showed at least a 10-fold decrease in relative expression of MMP-2 gene as compared to DMSO-LPS. Although the group exposed to TCDD alone showed less relative expression of MMP-2 than the negative control (DMSO), this difference was not statistically significant. The bars correspond to the mean of four samples (run in duplicate) ± standard error. The columns with same letters are not significantly different ($p<0.05$).

As defined in the figure, the immune stimulation with LPS caused approximately a 12-fold increase in MMP-2 mRNA, while pretreatment of the cells with TCDD dramatically blunted this response to an only 3-fold increase above controls. Fig. 5.6 shows that pre-incubation of HD11 cells with resveratrol, a competitive AhR antagonist, resulted in a nearly 4-fold reduction in the relative expression of MMP-2 [0.28 ± 0.06 (SD) vs. 1.0; $P<0.05$], in contrast with the group exposed to TCDD alone [0.9 ± 0.06 (SD) vs. 1.0]. Stimulation of the resveratrol-blocked TCDD pre-exposed cells with LPS

did not significantly alter the effect of resveratrol blockage followed by TCDD exposure [0.28 ± 0.06 (SD) vs. 0.39] (Fig. 5.6).

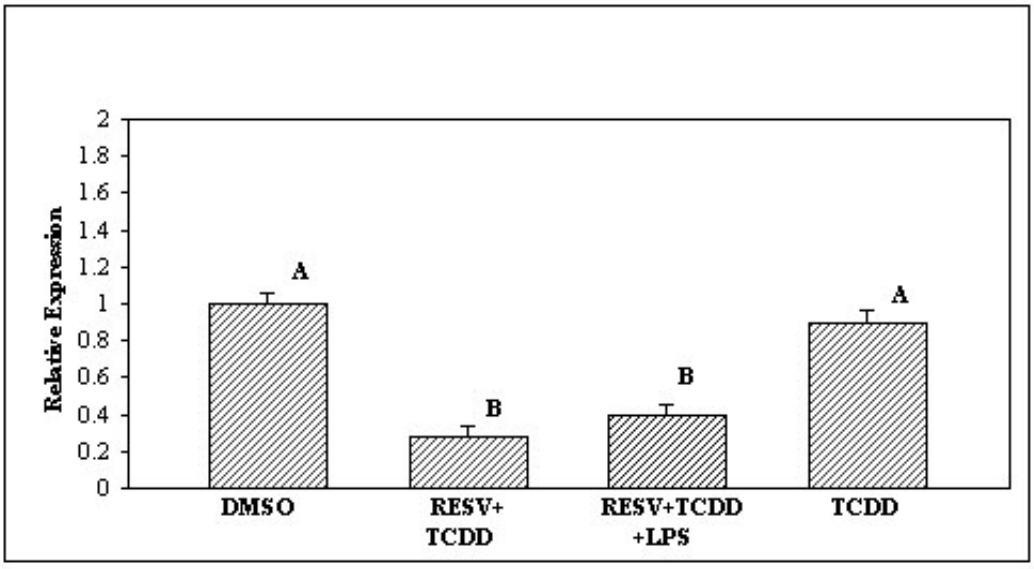

Figure 5.6. Effect of TCDD (10 nM) on the expression of MMP-2 in HD11 cells. Relative expression of MMP-2 following 6 h of LPS stimulation of cells exposed to 10 nM TCDD for 24 h.. Resveratrol at 5 μM was administered 1 h prior to TCDD. Each column represents the average (±SE) of ΔΔCT values obtained by real-time PCR correspondent to four samples in duplicate using β-actin as the calibrator. The bars correspond to the mean of four samples (run in duplicate) ± standard error. The columns with same letters are not significantly different ($p<0.05$).

As shown in Fig. 5.7, the effects of TCDD alone or in combination with LPS or resveratrol (Fig. 5.8) on the MMP-9 expression level were almost identical to the shifts observed for MMP-2. MMP-9 was upregulated (approximately 20-fold) by stimulation with LPS, and prior exposure to TCDD (10 nM for 24 h) dramatically blunted this response to approximately one third of the response of control cells.

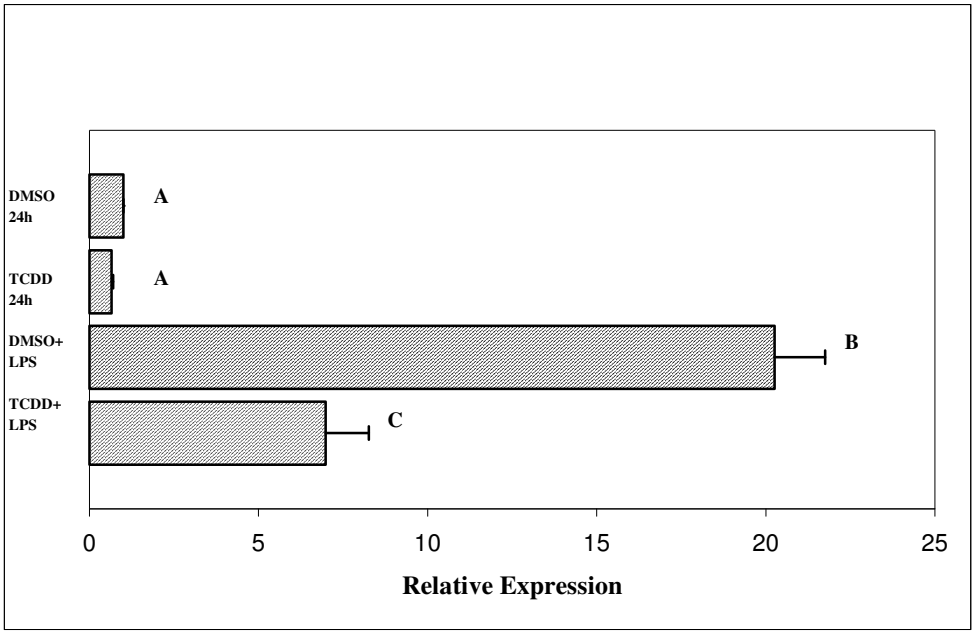

Figure 5.7. Effect of TCDD (10 nM) and LPS on the expression of MMP-9 in chicken macrophages (HD11). Real-Time PCR results after analysis using the ΔΔCT method. TCDD+LPS treated cells showed at least a 12-fold decrease in relative expression of MMP-9 gene than the LPS-DMSO. Although the group exposed to TCDD alone showed less relative expression of MMP-9 when compared to DMSO alone, the difference was not statistically significant. The bars correspond to the mean of four samples (run in duplicate) ± standard error. The columns with same letters are not significantly different ($p<0.05$).

After pre-incubation of the cells with resveratrol (Fig. 5.8), a 40% decrease in relative expression of MMP-9 [0.6 ± 0.09 (SD) vs. 1.0; $P<0.05$] was observed. The group exposed to TCDD-LPS showed a similar (approximately two-fold) reduction of the relative MMP-9 expression [0.48 ± 0.09 (SD) vs. 1.0; $P<0.05$] (Fig 5.8).

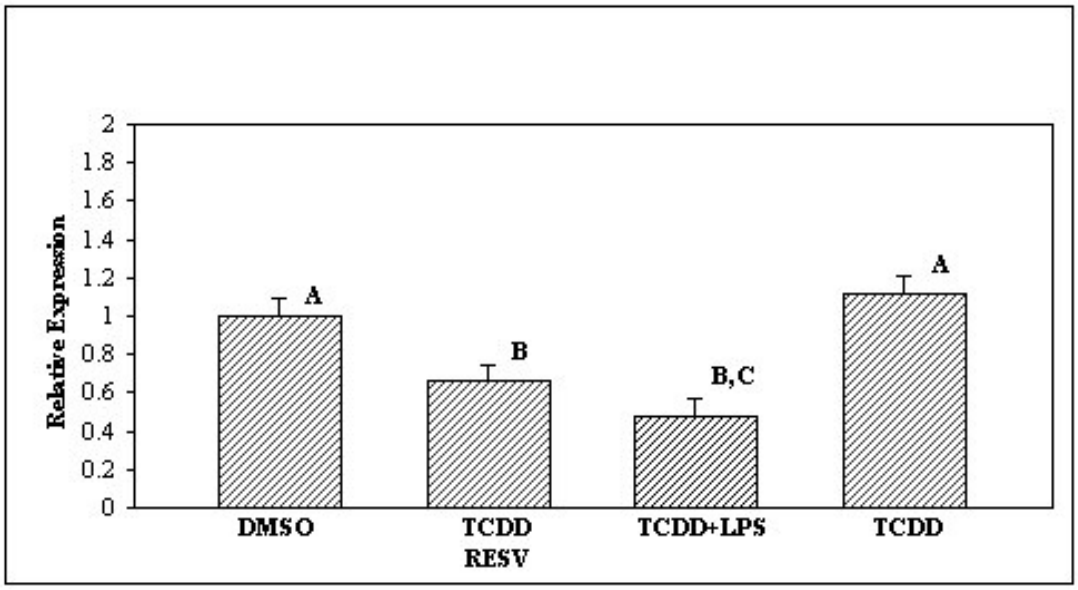

Figure 5.8. Effect of TCDD (10 nM) on the expression of MMP-9 in HD11 cells. Relative expression of MMP-9 after 24 h of 10 nM TCDD exposure following 6 h of LPS stimulation. Resveratrol at 5 μM was administered 1 h prior to TCDD. Each column represents the average (±SE) of ΔΔCT values obtained by real-time PCR correspondent to four samples in duplicate using β-actin as the calibrator. The bars correspond to the mean of four samples (run in duplicate) ± standard error. The columns with same letters are not significantly different ($p<0.05$).

Enzymatic activity of matrix metalloproteinases secreted by HD11 cells

To determine whether reduced mRNA levels of MMP-2 and MMP-9 observed in RT-PCR resulted in lower secreted MMP enzymatic activity, conditioned media from resting and stimulated HD11 cells exposed to 10 nM TCDD for 24 h were evaluated in gelatinolytic assays. In none of the experimental conditions examined was any increased MMP enzymatic activity observed in the media. On the contrary, when HD11 cells had been stimulated with LPS, or pre-exposed to TCDD followed by stimulation with LPS, small but significant reductions in the levels of MMP-2 and MMP-9 were observed

compared to vehicle or TCDD-exposed groups (Tables 5.2 and 5.3). Gelatinolytic activity is, indeed, more evident in the DMSO group than in the TCDD-LPS exposed group (Figs. 5.9 and 5.10).

Table 5.2. Relative density of MMP-2 bands on a gelatinolytic assay.

DMSO	DMSO+LPS	TCDD	TCDD+LPS
4.31 ± 0.04^{a}	3.45 ± 0.01^{b}	4.08 ± 0.12^{a}	3.51 ± 0.15^{b}

Values represent the mean density of three bands per experiment ± SE (n=3). *P<0.05. The gel was scanned using the Fluor-S MultiImager scanner (BIO-RAD). The image colors from the original scan were inverted so the clear bands observed in gelatin degradation were shown in black. The density of three bands per experiment was calculated using Quantity One 4.0 software (BioRad). The values are represented in relative units.

Table 5.3. Relative density of MMP-9 bands on a gelatinolytic assay.

DMSO	DMSO+LPS	TCDD	TCDD+LPS
4.09 ± 0.02^{a}	3.47 ± 0.06^{b}	3.82 ± 0.07^{c}	3.32 ± 0.12^{b}

Values represent the mean density of three bands per experiment ± SE (n=3). *P<0.05. The gel was scanned using the Fluor-S MultiImager scanner (BIO-RAD). The image colors from the original scan were inverted so the clear bands observed in gelatin degradation were shown in black. The density of three bands per experiment was calculated using Quantity One 4.0 software (BioRad). The values are represented in relative units.

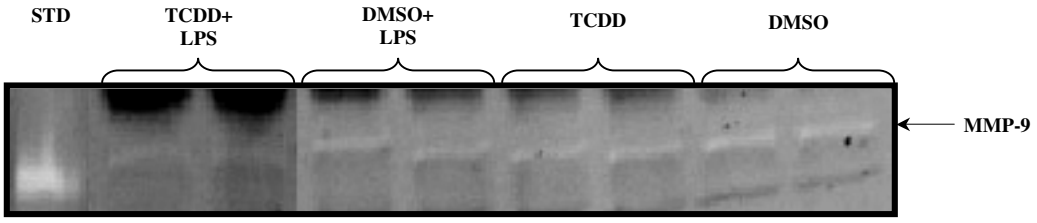

Figure 5.9. Gelatinase assay in HD11 cells (MMP-9). Cells previously exposed to TCDD (10 nM) and treated with LPS (10 µg/ml) showed reduction in gelatinolytic activity as compared to DMSO+LPS and DMSO alone. Increased gelatinolytic activity caused by MMP-9 is observed in the negative control group (DMSO only) than the TCDD alone and DMSO-LPS or TCDD-LPS. Two assays run in triplicate were included in this assay (the picture shows only two bands per treatment).

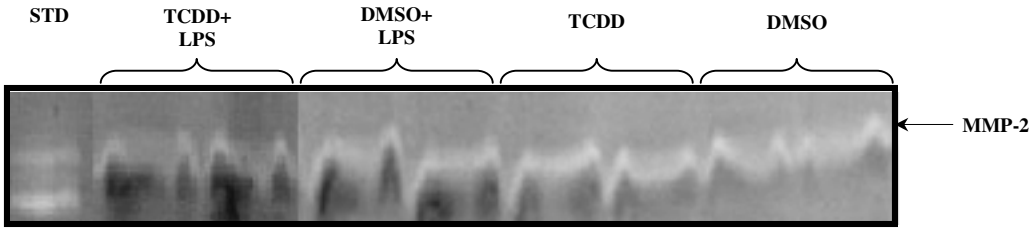

Figure 5.10. Gelatinase assay in HD11 cells (MMP-2). Cells previously exposed to TCDD (10 nM) and treated with LPS (10 µg/ml) showed reduction in gelatinolytic activity as compared to DMSO+LPS and DMSO alone. Increased gelatinolytic activity caused by MMP-2 is observed in the negative control group (DMSO only) than the TCDD alone and DMSO-LPS or TCDD-LPS Two assays run in triplicate were included in this assay (the picture shows only two bands per treatment).

Discussion

Although dioxins have previously been recognized as potent immunotoxicants (Kerkvliet, 2002), most of the immunosuppressive effects that have been documented concern the deleterious effects on lymphocytes (Holsapple et al., 1991; Hossain et al., 1998; Puebla-Osorio et al., 2004). However, evidence is gathering that xenobiotic AhR ligands also have considerable negative effects on various functional aspects of immune cells of the myeloid lineage. For instance, the differentiation of human blood monocytes into macrophages is inhibited by polycyclic aromatic hydrocarbons, such as benzo(a)pyrene (BP) (van Grevenynghe et al., 2003). In addition, TCDD was reported to suppress activation of dendritic cells by tumor necrosis factor-α and anti-CD-40 (Ruby et al., 2002). The main contribution of the present study is the identification of an aspect of the immunotoxic effects of dioxins on macrophages that has, to the best of our knowledge, not been previously described, namely, their capacity to degrade the extracellular matrix in response to an inflammatory stimulus. The connection between TCDD exposure and MMP function was suggested by preliminary cDNA microarray studies. Previous microarray data obtained from HD11 cells exposed to 1 or 10 nM TCDD for 6 and 12 h, showed a consistent downregulation of matrix metalloprotease 2 (MMP-2) (for results see chapter IV).

The first stage of our study aimed at validating the HD11 cell line for the study of the immunotoxic effects of TCDD at the level of the innate immune system. The presence of the myc-encoding MC29 virus in the chicken myelomonocytic HD11 cells (Beug et al., 1979) was shown to leave the expression and the biological activity of

MMP-9 and MMP-2 intact (Hahn-Dantona et al., 2000). In addition, the activation of the detoxification machinery of the cells, exemplified by the upregulation of CYP1A1 by more than one order of magnitude, clearly demonstrated the integrity of the downstream signaling pathway initiated by binding of TCDD to the cytosolic AhR (Fig. 5.2). These detoxification mechanisms appear to be more active in immune-stimulated than in resting macrophages, although immune stimulation *per se* does not increase CYP1A1 activity. In fact, a clear numerical decrease of CYP1A1 was observed in LPS-stimulated macrophages (Fig. 5.2). These data establish, for the first time, HD11 cells as a valid in vitro experimental model for the study of dioxins.

In general, limited information is available relative to the effect of TCDD on the activity of matrix metalloproteases such as MMP-2 and MMP-9, much less so in an immune context. As far as the effects of dioxin on MMPs has been described, the molecular mechanisms underlying the effect of TCDD on the expression of matrix metalloproteases, in particular MMP-2 and MMP-9, are not fully understood. Recent studies described that TCDD exposure results in increased expression of matrix metalloprotease 1 (MMP-1, interstitial collagenase) in keratinocytes. This effect is further enhanced by co-treatment with all-trans retinoic acid expression, suggesting interaction between the AhR pathway and the retinoic acid pathway (Murphy et al., 2004).

Matrix metalloproteases are best known for their role as effectors or executioners in the process of inflammation, but also in the context of tumor invasion and metastasis (McQuibban et al., 2000; Theret et al., 2001). Although T-cells secrete limited amounts

of MMPs in order to prepare connective tissue matrices for T cell chemotaxis across basement membranes and through tissues, macrophages are the major producers of metalloproteases in the immune system (Goetzl et al., 1996). In response to physiological needs, but also in pathological situations, these MMPs enable macrophages to modulate matrix turnover.

To further study the effects of TCDD on the expression of MMP-2 and MMP-9 , real-time PCR was carried out for these two metalloproteases, and for the CYP1A4 and AhR genes, under both resting and LPS-stimulated conditions. To test whether the toxic effect of TCDD on HD11 cells was also affecting the secretion of both MMP-2 and MMP-9, gelatinolytic assays (zymograms) were performed.

TCDD caused a discrete, but statistically non-significant, downregulation of the mRNA for AhR 24 h after exposure (Fig. 5.3). In contrast, activation with LPS (10 μg/ml), caused an increase of relative expression of the AhR of more than 7-fold. This suggests that activated macrophages are more sensitive to TCDD exposure than resting macrophages. The combination of pre-exposure to TCDD followed by LPS activation further increases the transcription of the AhR message, suggesting a synergistic effect that ultimately may lead to a chain reaction of downstream molecules. Similar upregulation of the AhR gene was observed in B cells (CH12.LX) exposed to TCDD and LPS, reaching a maximum peak after 6 h of LPS exposure (Sulentic et al., 1998).

Protein levels of the AhR were not determined in the present study, but other studies describe a rapid degradation of AhR protein, through the ubiquitin-proteasome complex, after exposure to TCDD (Giannone et al., 1998; Pollenz, 1996). When the

cells were pretreated with resveratrol, all of the above effects of LPS, and of the combination TCDD-LPS, were lost (Fig. 5.4), as the only effect observed was a modest downregulation of the AhR message. This shows that the AhR plays a role in the upregulation of its own message by TCDD in stimulated macrophages. It would have been interesting to determine if resveratrol prevents the upregulation of the AhR message by LPS activation alone, but this experimental condition was not included in our study. However, such effect would assume that resveratrol can block an effect that is mediated through Toll-like receptor 4 (TLR4) and/or TLR2 (Dil and Qureshi, 2002a; Dil and Qureshi, 2002b; Fukui et al., 2001), which has not been previously reported and remains to be determined in an experiment that would include LPS activation first followed by TCDD exposure.

As mentioned above, (Andersson et al., 1991; Bjerke and Peterson, 1994; Hayashi et al., 1995) the MMP-2 (72-kD) and MMP-9 (92-kD) are members of a group of secreted zinc metalloproteases that degrade the collagens of the extracellular matrix (Hahn-Dantona et al., 2000; Nagase et al., 1992). Both metalloproteases play important roles in processes ranging from metastasis (tumor invasion) to inflammation and chemotaxis (Goetzl et al., 1996). Under the conditions used in this study, TCDD alone caused a decrease in the expression of MMP-2 in HD11 cells, although this numerical trend was not statistically significant. This would suggest that the downregulation of MMP-2 observed in previous cDNA microarray studies may have been slightly overestimated. However, TCDD induced a highly significant blunting (i.e. a 3-fold reduction) of the increase in MMP gene expression that normally follows stimulation

with LPS in macrophages (Figs. 5.5 and 5.7). Indeed, LPS-induced upregulation of MMP-9 expression in cells not previously exposed to TCDD was 20-fold, while pre-exposure to TCDD caused this increase to drop to 7-fold. This demonstrates that pre-exposure to TCDD causes a disruption of the effector and regulatory activities of both MMP-2 and MMP-9 in macrophages in response to inflammation.

To test whether the decrease in relative expression of MMP-2 and MMP-9 was AhR-mediated, the cells were preincubated with resveratrol for 1 h. Treatment with resveratrol *per se*, however, is known to inhibit the gelatinase activity of MMP-9 in U937 cells, both at the protein and at the mRNA level (Li et al., 2003). This makes it impossible to conclude whether the decrease observed in the combinations resveratrol-TCDD and resveratrol-TCDD-LPS (Figs. 5.4 and 5.6) is caused, or by resveratrol or by AhR-independent TCDD mechanisms. The use of a different AhR antagonist (such as alpha naphthoflavone) or gene silencing technology is needed to elucidate whether the observed changes in relative expression and gelatinase activity are AhR-mediated.

The effect of pre-exposure to TCDD followed by an LPS stimulus was also assessed in a gelatinolytic assay (Fig. 5.10). Surprisingly, densitometry data obtained from scanned gels did not reveal any increases in secreted MMP activity, but rather showed a significant reduction of gelatinase activity in response to an LPS stimulus, with or without pre-exposure to TCDD (Tables 5.2 and 5.3).

This finding contrasts with the expected increase in gelatinase activity in LPS-stimulated macrophages, suggesting that 6 hours after LPS stimulation is probably too early to observe increases in gelatinase activity, when in most cases this is observed after 24 or 48 h of LPS stimulation (Warner et al., 2004).

In conclusion, the presented data show for the first time that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) causes downregulation of LPS-stimulated matrix metalloprotease MMP-2 and MMP-9 transcription levels in chicken macrophages.

Although our results do not show quantitative data of reduction of the amount of MMP protein that is being secreted in response to an inflammatory stimulus we do have visual evidence that this is highly probable. In view of the potential consequences of TCDD exposure for this aspect of innate immune function, this remains an important topic for future investigation.

CHAPTER VI

GENE PROFILE OF THE EMBRYONIC BURSA OF FABRICIUS: MODULATED BY 2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN (TCDD) IN OVO

Overview

We have used a commercially available high density cDNA microarray to analyze the transcriptional profile induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in the developing chicken embryo. The array was produced at the Fred Hutchinson Cancer Research Center (FHCRC), and consisted of 13,007 clones that included different sources of chicken cDNA libraries and control features. The annotation file is available at: ftp://milano.fhcrc.org/ArrayLab/chicken13k/annotation/. Thirty Plymouth Rock fertile eggs were injected with 0.3 pmol TCDD/g of egg, dissolved in corn oil, and 15 eggs received an equal amount of corn oil as a negative control. At day 18 of embryogenesis, the bursa of Fabricius was excised from each embryo. Total RNA from individual bursae from the TCDD-exposed and the negative control embryos, was extracted. Two batches of pooled total RNA from the exposed bursae were used for hybridization onto two different array slides, each time against a pool of control RNA. Scanning and image processing were carried out using a GenePix 4000 Microarray scanner and GenePix 4.0 respectively (Axon Instruments, Inc., Union City, CA). Data analysis was performed using GENESIS (http://genome.tugraz.at). The analysis included internal Loess normalization to correct for dye effect, filtering of bad

spots and logarithmic transformation of the ratios. Log2 values of median ratios were used for analyses of gene expression profiles. Upon filtering, approximately 106 genes were found to be significantly up- or down-regulated. These genes were further analyzed by hierarchical clustering (HCL) and dendrogram formation. Various genes involved in regulation of the cell cycle and development were significantly down-regulated, and could thus help explain the bursal weight loss and atrophy in TCDD-exposed chicken embryos. Potentially the most interesting working hypothesis so far generated by this microarray study stems from the observed down-regulation of the cytokine-like protein C17, a cytokine expressed by a rare population of hematopoietic stem/progenitor cells in humans. This cytokine has never been reported in the context of TCDD intoxication, and its downregulation during a crucial developmental stage of central humoral immune system could potentially result in deficiencies of the humoral immune system postnatally.

Introduction

The classic dioxin, TCDD, causes a myriad of biological effects that are mediated almost entirely through the activation of the aryl hydrocarbon receptor (AhR) (Schmidt et al., 1996). The physiological aspects of the receptor, as well as the pathological consequences of TCDD toxicity, were described in chapter I of this dissertation. In this chapter, we describe the toxic aspects of TCDD on the developing avian immune system, more specifically on the gene expression profile of the bursa of Fabricius. Numerous reports have demonstrated the immunosuppressive effects of

TCDD at the humoral and cellular immunity levels (Holsapple et al., 1991). The gross effects of TCDD on the chicken bursa of Fabricius, the primary humoral lymphoid organ of the chicken, have been extensively studied. Various reports show that TCDD and its congeners cause inhibition of lymphoid development, atrophy (Andersson et al., 1991), and decrease of bursal weight (Powell et al., 1996) in the developing chicken bursa of Fabricius. It is suggested that the inhibition of the lymphoid development in the bursa is caused by the effects of TCDD on the bursal microenvironment (i.e., the bursal stroma), resulting in a decrease in the attraction of stem cells and/or in the capacity to induce proliferation of the colonizing cells (Nikolaidis et al., 1990). Another possible explanation for the weight loss of the bursa of Fabricius could be related to the induction of apoptosis in primary B-cells (DT40) by TCDD (Puebla-Osorio et al., 2004). Much of the earliest characterization of the AhR comes from the chick embryo model (Walker et al., 2000), likely because the chicken embryo (*Gallus gallus*) is one of the most sensitive vertebrates to TCDD-induced developmental toxicity (Allred and Strange, 1977; Powell et al., 1996). The chicken embryo has been used to characterize the developmental and teratogenic effects of TCDD and related chemicals (Allred and Strange, 1977; Cheung et al., 1981; Rifkind et al., 1984). TCDD-inducible cytochrome P450 regulation (Bentivegna et al., 1998; Gilday et al., 1998; Hamilton and Bloom, 1983; Hamilton et al., 1983), AhR structure-activity relationships (Brunstrom et al., 1991) and AhR binding affinity and DNA binding characteristics (Sanderson and Bellward, 1995). In addition to the initial extensive employment of the chick embryo for elucidating the mechanism(s) underlying TCDD teratogenicity and other effects, the molecular characteristics of the

chicken AhR were recently elucidated (Walker et al., 2000). These authors showed that the chicken AhR protein sequence is highly conserved among mammalian and avian species and that it functions in a manner analogous to the mammalian AhR. The basic helix-loop-helix domain (chicken residues 8-77) exhibits > 91 % amino acid identity with mammalian AhR proteins. Also the PAS amino acid residues (chicken residues 131-345) are well conserved with the mammalian AhR (81-86%). Furthermore, these investigators showed that the chicken AhR dimerizes with human AhR nuclear translocator (Arnt) and binds to the mammalian dioxin-response element (DRE) in a ligand-dependent manner. Finally, cytochrome P4501A4, the avian analogue of CYP4501A1 (Gilday et al., 1998), was highly induced by TCDD in a subset of tissues expressing AhR. Clearly, all these findings strengthen the use of the avian embryo as a model to help elucidate the molecular, morphological and functional effects of TCDD on embryonic development.

A comprehensive study of the transcriptional profile of the embryonic bursa of Fabricius exposed to TCDD has not been undertaken so far, due to the limitations in the availability of a dedicated array, including extensive annotation of the spots on the array.

This type of longitudinal study has the potential to lead to a better understanding of the transcriptional mechanisms underlying the immunotoxicity of TCDD and its biochemical and physiological consequences.

We have used a commercially available high density DNA microarray developed at the Fred Hutchinson Cancer Research Center (FHCRC) to start the development of a database of genes whose expression is affected by TCDD. In contrast to the chicken

immune cDNA array described in chapters II and IV, which contained approx. 4000 spots, this time an upgraded version consisting of approx. 13,000 clones was employed.

Chicken embryos were exposed to TCDD via the egg yolk at six days of embryonic development, i.e. before the stem cells enter the bursa of Fabricius anlage; in chickens, this event occurs between 7.5 and 14 d of embryogenesis (Glick, 1995). In previous chapters, cDNA microarray analyses have proven useful for the identification of differentially expressed genes following exposure of immune cell lines to TCDD in vitro. The present study is different in that the gene expression profiles that were observed are not those of a homogeneous cell population (as was the case in previous chapters) but rather the combined profiles of B-lymphocytes and various bursal stromal cell populations.

Materials and Methods

Chicken 13k cDNA microarray

The array was an upgraded version of the previously described 4k cDNA microarray and consisted of 13,007 clones, including additional control features, and different sources of chicken cDNA libraries. The annotation file is available online at: ftp://milano.fhcrc.org/ArrayLab/chicken13k/annotation/.

The values for each channel were obtained using the correction for variation in the local background. The values spanned the maximum fluorescent dynamic range of detection, from over 65,000 counts down to background count levels. A consistent low

average of local background signal was obtained in both channels, with no appreciable differences between different spatial blocks (Neiman et al., 2001; Neiman et al., 1991).

Chicken embryos, chemical treatment and sample preparation

Forty-five Plymouth Rock fertile eggs were incubated at 37.5 ºC and 70 % humidity in the Laboratory of Molecular Toxicology at the University of New Mexico Health Sciences Center (courtesy of Dr. Mary Walker). Thirty embryonated eggs were injected via the yolk sac on day 6 of embryonic development with 0.3 pmol TCDD per gram of egg (Ivnitski-Steele and Walker, 2003). TCDD was dissolved in corn oil and 15 eggs received an equal amount of corn oil as a negative control.

At day 18 of embryogenesis, the embryos were euthanized and the bursa of Fabricius excised from each embryo. The bursae were individually flash frozen in liquid nitrogen for future use. Total RNA was extracted individually from bursae of TCDD-exposed embryos and from negative control bursae, using the RNeasy® mini kit (Qiagen, Valencia, CA). Total RNA concentration was determined by OD_{260}. Two pools of 10 samples of total RNA each, from TCDD-exposed samples, and a single pool of 10 samples from the control group were created.

Sample labeling and hybridization

Labeling of cDNA samples, preparation of microarrays, and hybridization reactions were performed, as a custom service, by the FHCRC. Here, for informative purposes, the techniques are briefly described. The total extracted RNA was linearly

amplified using a T7 promoter-based method (Ambion, Inc., Austin, TX) and random prime labeling. A minimum of 30 μg of each pool of total mRNA was used, and then transformed into cDNA. Target cDNAs from control RNA samples were labeled by reverse transcription using Cyanine-3 (Cy3) and the TCDD-treated samples were labeled with Cyanine-5 (Cy5). The coupling of both fluorophores was carried out using an established amino-allyl coupling procedure. The Cy3- and Cy5-labeled cDNA samples were combined and co-hybridized to the array for 16 hrs at 63°C, followed by a sequentially stringent washing procedure (Fazzio et al., 2001).

Image scanning and data processing

Hybridized slides were scanned using a GenePix 4000 Microarray scanner (Axon Instruments, Inc., Union City, CA). Two 16-bit tiff images were collected for each array corresponding to 532 nm (Cy3) and 635 nm (Cy5) excitation wavelengths. Image analysis was performed using GenePix 4.0 software (Axon Instruments, Inc.). Raw values were obtained as *.gpr file, and were processed using GENESIS (http://genome.tugraz.at/Software/GenesisCenter.html). Twenty percent of non-significant spots (similar readings as the background) were filtered out and Loess normalization for adjustment of dye effect was performed. Logarithmic transformation was calculated from the resulting expression ratios.

Determining repression or induction

The method for determining the expression ratios has been explained in previous chapters (chapters II and IV). The expression ratio is calculated as a decimal value (*e.g.* 0.2 for a 5-fold reduction), and the decimal value is then transformed into logarithmic (base 2) scale, which makes it easier to visualize induction versus repression (Gasch et al., 2000).

$$\textit{Ratio: red : green} \quad \rightarrow \quad \textit{red} \div \textit{green} \quad \rightarrow \quad \log_2 (\textit{red} \div \textit{green})$$

If the expression of the experimental sample is greater than the control, the ratio will be greater than one, and then the final number after logarithmic transformation will be positive. It is very important to emphasize that the artificial colors presented in the dendrograms and do not represent the initial fluorescence from the mRNA hybridization experiment. In a typical scale, a black spot on the array would indicate that equal amounts of red and green fluorescence were observed on the original spot, thereby giving an equal expression ratio of 1:1, and subsequently a logarithmic value of 0.

Clustering

The process of separating a set of objects, according to their similarity, into several subsets, is called clustering (Gilbert et al., 2000). In general, two strategies are possible: supervised (based on existing knowledge) and unsupervised clustering. In supervised clustering, existing biological information is used to guide the classification of genes affected by chemical treatment. We have used hierarchical clustering, which is

referred to the method that identifies a small group of genes that share a common pattern of expression and that constructs a dendrogram in a sequential manner using a ranked series of clusters (Schena, 2003). Even though we have also utilized k-means clustering (non-hierarchical method that assigns each gene expression datum to a cluster based on its expression profile, and then repeats the process until every datum point has been placed in a cluster), in our final analysis here we made use of the hierarchical clustering approach.

Analysis of microarray data

Quantitation of data is a computational process by which numerical values are obtained from microarray data files, and involves absolute quantification, ratio calculations, and normalization.

Microarray scanners acquire data as tagged image file format (*.tiff), which is a two-dimensional intensity map of the microarray surface, with fluorescence signals stored in square bins known as pixels. Each spot on the microarray, which normally represents an individual gene, contains multiple pixels each of which contains numerical information. In the present investigation, we made the effort to comply with the minimum information about a microarray experiment (MIAME, http://www.mged.org/Workgroups/ MIAME/miame.html). The only criterium that was not satisfied was the requirement for “dye swap” (where the dyes are swapped between samples from controls and exposed birds), because of time limitation as well as resources.

Results

Treatment with 0.3 pmol TCDD/g of egg in chicken embryos resulted in a change of gene expression of at least 106 genes from an approximate 13,000 annotated clone sequences. Fluorescent ratios on the arrays were obtained by dividing the normalized absolute expression of the TCDD-exposed sample (Cy5, red) by the absolute expression of the negative control sample (Cy3, green). The quantification of both fluorophores (red and green) is carried out by image scanning. The ratios were logarithmically transformed, and these values were used for generating hierarchical clustering. The criterium to select significantly shifted genes was based on a deviation of the two standard deviations from the mean (of all clones on the array); the cut off point of the log2 ratio was established at 0.75 above and 0.75 below zero.

The genes were then clustered using hierarchical clustering according the their expression value (Figs. 6.1, 6.2 and 6.3). Thus, six clusters were created; cluster A included all up-regulated genes; cluster B included genes that were up-regulated on the first array and down-regulated on the second array. Clusters C-F included all down-regulated genes. Of the listed genes, 20 genes were significantly up-regulated and 71 were significantly down-regulated on duplicate arrays. When genes were up-regulated on the first (of duplicate arrays) and down-regulated on the second array, (a cluster of 15 genes; B, top right in Fig. 6.2) these genes were ignored during further interpretation.

Table 6.1. Selected genes from analysis of microarray of embryonic bursa of Fabricius exposed to TCDD (0.3 pmol/g of egg). Induced changes in expression involve apoptosis, metabolism, and cell cycle regulation, among others.

Gene Bank Number	Gene name	Induced		Repressed		Function	Reference
		A	B	A	B		
607930	Cytokine-like protein C17			-0.8	-1.2	Cells that bear the CD34	(Liu et al., 2000)
O14529	Homeobox protein Cux-2			-1.5	-0.7	Development	(Quaggin et al., 1996)
Q12947	Forkhead protein			-1.5	-0.8	Cell cycle	(Medema et al., 2000)
P53355	Death assoc. protein kinase (DAP-1)			-0.8	-0.8	Promotes cell survival	(Jin et al., 2001)
O08550	Trithorax homolog-2			-2.1	-2.9	Development	(Crosby et al., 1999)
15040845	PK-Cepsilon	3.4	2.2			Protects vs. ischemic cell death	(Jung et al., 2004)
601641	Acyl-CoA oxidase	1.3	2.0			Lipid metabolism	(Baumgart et al., 1996)

Notes for treatments: A) Bursa first array from duplicate arrays; B) Bursa second array from duplicate arrays

Generally speaking, in ovo exposure to TCDD largely caused significant down-regulation of the majority of genes that displayed a significant shift. Indeed, 71 genes, subdivided into 4 clusters were downregulated, whereas only 20 genes were upregulated.

Table 6.1 shows the data mining results for a small selection of up- and down-regulated genes. The gene in the list (Cytokine –like protein C17) is the chicken homolog of a cytokine that has been reported in humans to be typically produced by a rare population of human bone marrow (BM) and cord blood (CB) mononuclear cells bearing the CD34 surface marker (CD34+) function as hematopoietic stem/progenitor cells (Liu et al., 2000).

The exact function of this protein that has the structural features of a cytokine has not been described so far. The next four genes in the list (homeobox protein Cux-2, forkhead protein, death-associated protein kinase and trithorax homolog-2) all seem to be involved in cell cycle and developmental regulation (Crosby et al., 1999; Jin et al., 2001; Medema et al., 2000; Quaggin et al., 1996). A strongly (4- to 8-fold) upregulated gene is PKC-epsilon. The PKCepsilon-JNK/p38 MAPK signaling module has been shown in cardiac myocytes to contribute to chemical hypoxia-induced cell death. In other contexts, PKCepsilon was reported to have a protective effect against ischemic death. This suggests that PKCepsilon can mediate alternative signals, i.e., beneficiary or deleterious signals, depending on the cell type, intensity, and/or type of injury. Finally, Acyl-CoA oxidase is an enzyme involved in lipid metabolism (Baumgart et al., 1996), but the functional significance of this type of enzyme in the context of the bursa of Fabricius is as yet unclear.

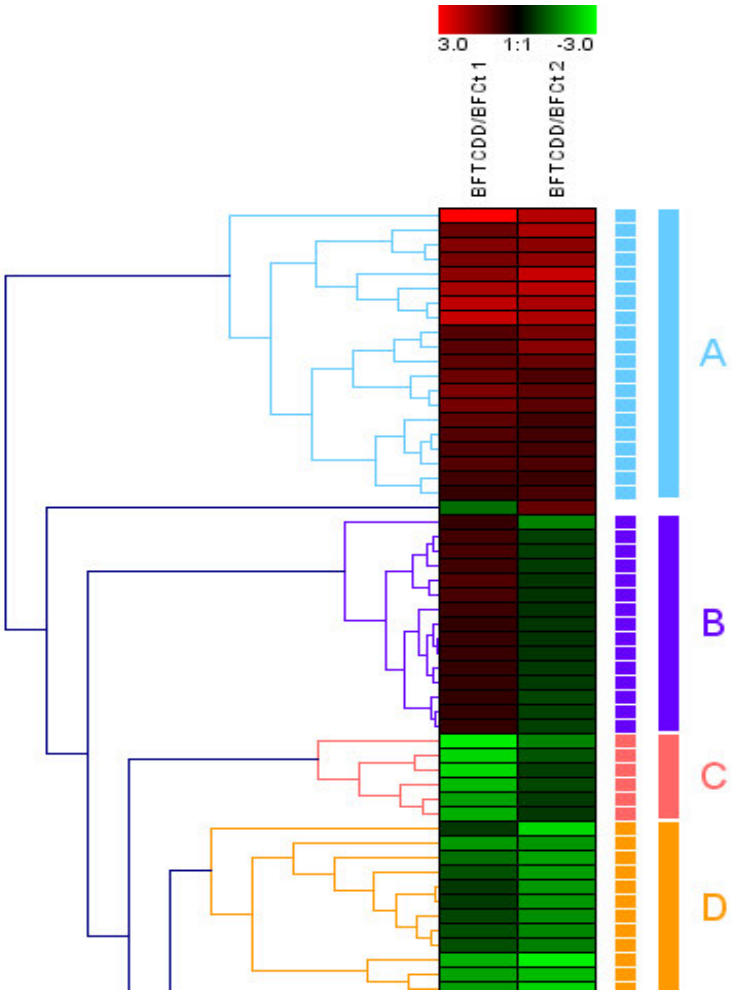

Figure 6.1. Cluster analysis of microarray data from embryonic bursa of Fabricius exposed to TCDD from day 6 to day 18 of incubation. The tree (dendrogram) is the result of hierarchical clustering analysis using average linkage between genes. The analysis was performed using GENESIS microarray software (http://genome.tugraz.at/Software/ GenesisCenter.html).

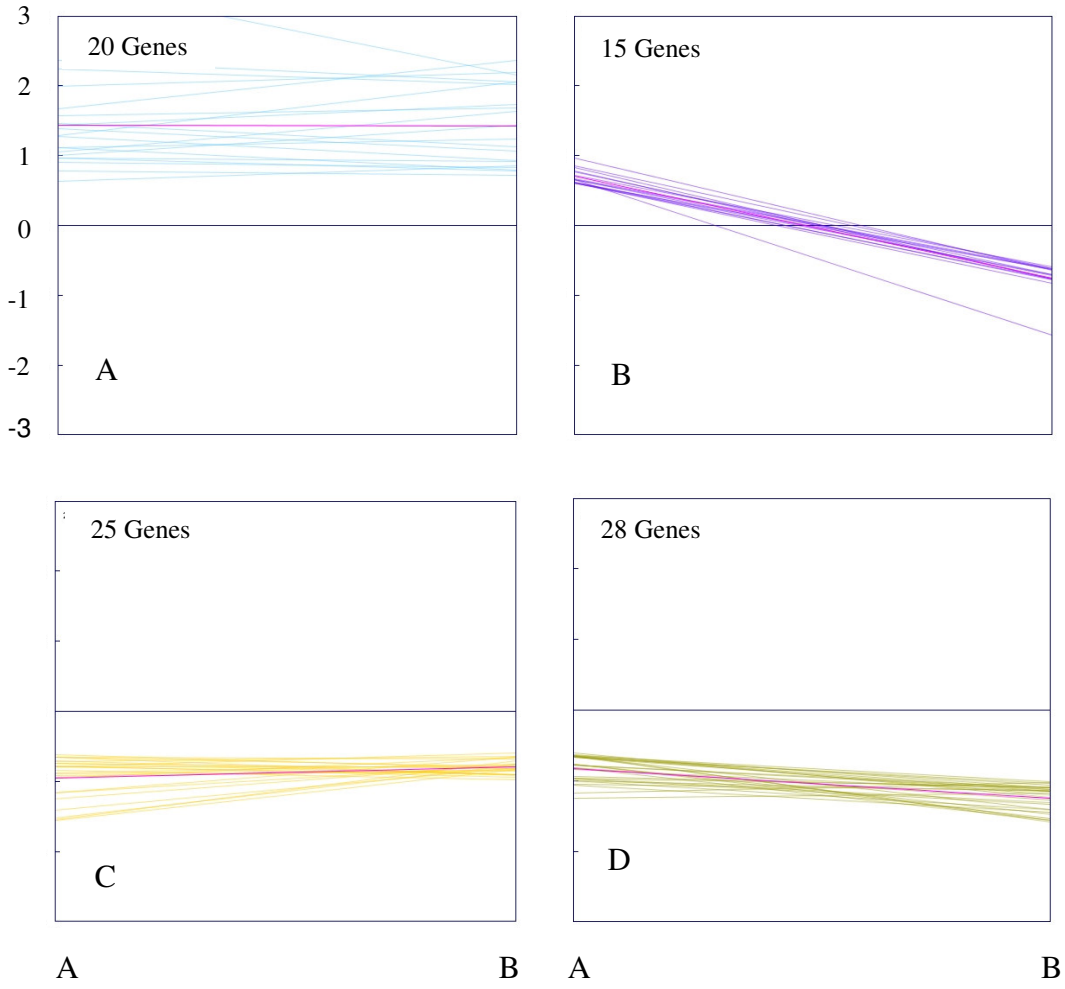

Figure 6.2. Gene expression profile using hierarchical clustering (HCL) (Schena, 2003) in embryonic bursa of Fabricius exposed to TCDD from day 6 to day 18 of incubation. The HCL method identifies a small group of genes that share a common pattern of expression; it constructs the dendrogram in a sequential manner using a ranked series of clusters. The positive numbers indicate the up-regulated genes, and the negative indicate the down-regulated genes. A and B refer to duplicate arrays representing the same treatment.

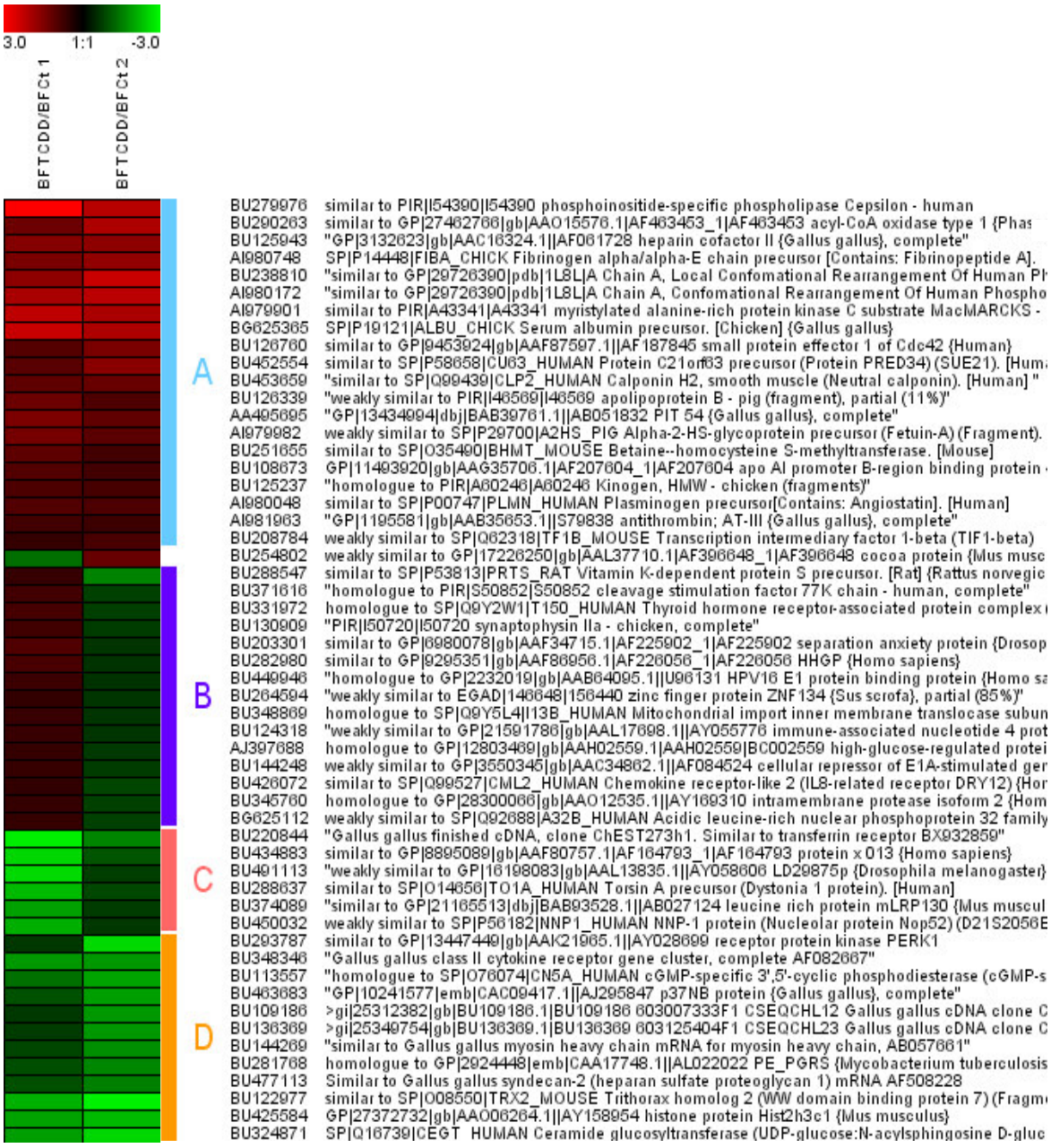

Figure 6.3. Cluster analysis of microarray data from embryonic bursa of Fabricius exposed to TCDD from day 6 to day 18 of incubation. The genes were classified according to their average expression. This panel is part of the original dendrogram which consisted of 6 clusters (A to F), cluster A shows the top up-regulated genes. Colored columns shows the treatment groups, the single bar identifies each cluster, the third column shows the GeneBank accession number (as far as available), and the fourth column shows the description for each gene, as far as it is known.

Discussion

cDNA microarray analysis provides a powerful hypothesis-generating tool. Microarray analyses of B-cells and macrophages exposed to TCDD in vitro have been used in the context of this dissertation to generate a working hypothesis that was afterwards successfully corroborated and refined. The major obstacle in studies like these is obviously the overflow of data that are generated by microarray studies, even if the experiments are kept as simple as possible. In this respect, the study performed in this chapter poses two additional challenges. First, the microarray that was used did not contain 4,000 but 13,000 features and second, the biological system was not a cell line propagated in vitro, but a complete immune organ dissected after in vivo exposure of chicken embryos. Surprisingly, the list of genes that was selected by the software algorithms (GENESIS) as having significantly shifted in their expression levels, was not noticeably more extensive with this 13k microarray when compared to the list generated by the 4000k array. The most plausible reason for this is the fact that the additional 9,000 clones were not immune organ-specific genes. Clones resulting from other organs (such as adipocytes, liver, pituitary etc.) had indeed been added to the original 4k array.

In addition, as mentioned above, the biological variation within a living system is substantially higher than in a simple cell line system (consisting of cells that are essentially genetically identical). Each data point in the present study reflected the average value of 10 (outbred) animals. These 10 animals may have reacted to the experimental treatment to a different extent, or even in opposite directions. This is most clearly illustrated by the “B” cluster, showing significant differences between two

groups of 10 animals each that underwent the exact same experimental treatment. In addition, the RNA sample was the product of different cell populations, some of which may have reacted and others not, or some of which may have reacted in opposite directions.

In spite of the above limitations, interesting hypotheses can already be distilled from the data set presented in this chapter. The significant down-regulation of genes involved in regulation of the cell cycle and development (homeobox protein Cux-2, forkhead protein, death-associated protein kinase and trithorax homolog-2) may further corroborate the phenomenon of enhanced apoptosis of developing B-cells in response to TCDD exposure as described in Chapters II and III. Potentially even more interesting, down-regulation of the cytokine-like protein C17, a cytokine expressed by a rare population of hematopoietic stem/progenitor cells, has never been reported in the context of TCDD intoxication. Downregulation of this cytokine could result in deficiencies of the humoral immune system and might thus offer a mechanism for immune deficiency in the postnatal animal as a consequence of pre-natal exposure to TCDD. Sensitivity of the immune system, to TCDD, in both young and adult chicken is caused by a dose-dependent suppression of B-cell proliferation and induction of CYP450 enzymes. Hence, the chicken is considered an excellent model to study this cytokine in the scope of the humoral immune system because only birds have an anatomically delineated central B-cell organ such as the bursa of Fabricius, and because prenatal administration of TCDD has proven easy and straightforward.

CHAPTER VII

SUMMARY

The overall objective of this project was to investigate the immunotoxicity of 2,3,7,8-tetra-chlorodibenzo-p-dioxin (TCDD) in an avian species, i.e. the chicken. The general approach to attain this objective was to first use cDNA microarray analyses as a hypothesis-generating technology, in order to acquire a comprehensive view on the gene alterations caused by exposure to TCDD. cDNA microarray analyses were performed in three different paradigms: (1) after exposure of the chicken pre-B-cell line DT40 to various doses of TCDD; (2) after exposure of the chicken macrophage line HD11 to TCDD; and (3) after in ovo injection of TCDD in chicken embryos at 6 days of incubation, followed by sampling of the bursa of Fabricius at 18 days of incubation.

In each of these cases, the obtained transcriptional profiles could be used to generate specific working hypotheses with regard to the potential mechanism(s) underlying the immunotoxicity of TCDD in birds. In the first case, the hypothesis was that TCDD could shift the balance between pro- and anti-apoptotic genes to the extent that it would lead to increased apoptosis in pre-B-cells, and that this could be an explanation for the bursal atrophy observed in previous phenomenological studies. In the second case, the working hypothesis was that TCDD might downregulate the expression of the matrix metalloprotease genes, and that is a previously unknown aspect of the negative effect of TCDD on innate immune function. In the third (in ovo) model, the data suggested that several genes involved in the regulation of the cell cycle and cell

development were downregulated. More interestingly, a cytokine (cytokine-like protein C17) typically produced by a small set of hematopoietic stem cells in the bone marrow in humans, was shown to be down-regulated, and this could lead to humoral immune deficiency later in live.

The second phase of our approach was to use quantitative (real-time) PCR to verify and refine the hypotheses generated by the microarray analyses. The two first hypotheses were proven valid. The third hypothesis has not being tested. It is important to note that each of the microarray data sets potentially holds more promise than might appear from this study. Indeed, the abundance of data generated by microarray studies is at the same time the strength and the weakness of the technology. The conclusions of this type of studies only reveal themselves after intensive mining of the raw data, so it is impossible to tell whether a microarray study was successful before days and weeks have been spent on the analysis of the raw data. Clearly, the microarray data sets generated in the three different paradigms have not been completely exhausted. In hindsight, it now appears that the second model (in vitro exposure of macrophages to TCDD) has yielded the most significant and promising microarray data sets.

Nevertheless, more time needs to be spent on the analysis of the results obtained from the in vivo study. Although the system is much more complex than an in vitro study, it is also the closest to the real life situation. It would also be most interesting to be able to follow up the prenatally TCDD-exposed birds during the postnatal stage, in order to test the hypothesis that prenatal TCDD exposure may lead to postnatal immune deficiencies. This, however, will be the focus of future investigations.

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